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(54) **Title:** COMPOSITIONS AND METHODS FOR INDUCING AN IMMUNE RESPONSE IN A MAMMAL AND METHODS OF AVOIDING AN IMMUNE RESPONSE TO OLIGONUCLEOTIDE AGENTS SUCH AS SHORT INTERFERING RNAS
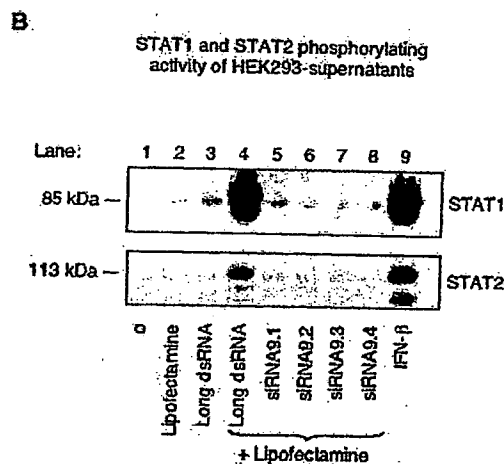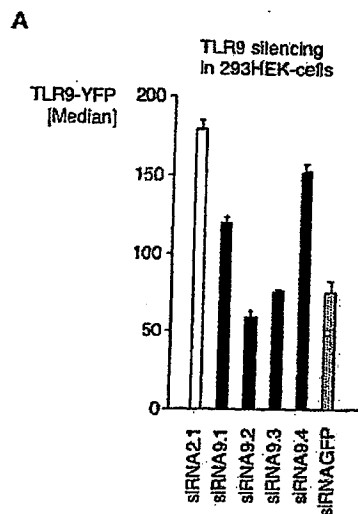
(57) **Abstract:** This invention provides oligonucleotide agents that modulate an immune response by stimulating IFN production and methods of using such agents for therapeutic treatments of mammals.

COMPOSITIONS AND METHODS FOR INDUCING AN IMMUNE RESPONSE IN A MAMMAL AND METHODS OF AVOIDING AN IMMUNE RESPONSE TO OLIGONUCLEOTIDE AGENTS SUCH AS SHORT INTERFERING RNAs

RELATED APPLICATIONS

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This application claims the benefit of U.S. Provisional Application No. 60/634,849, filed December 9, 2004, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

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This invention relates to the field of immunotherapy and drug discovery by providing sequence specific oligoribonucleotide agents that are capable of inducing an immune response in a subject as well as a method for avoiding sequence specific immune responses to RNAi agents.

BACKGROUND

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Double-stranded RNA molecules (dsRNA) can block gene expression by virtue of a highly conserved regulatory mechanism known as RNA interference (RNAi). Briefly, the RNA III Dicer enzyme processes dsRNA into small interfering RNA (also sometimes called short interfering RNA or siRNA) of approximately 22 nucleotides. One strand of the siRNA (the "antisense strand") then serves as a guide

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sequence to induce cleavage of messenger RNAs (mRNAs) including a nucleotide sequence which is at least partially complementary to the sequence of the antisense strand by an RNA-induced silencing complex RISC. The antisense strand is not cleaved or otherwise degraded in this process, and the RISC including the antisense strand can subsequently affect the cleavage of further mRNAs.

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The process of posttranscriptional dsRNA-dependent gene silencing is commonly referred to as RNA interference (RNAi) (Tuschl, T. *Chembiochem* 2, 239-45 (2001), Zamore, P.D. *Science* 296, 1265-9 (2002), Hannon, G.J. *Nature* 418, 244-51 (2002)). It has been proposed that eukaryotes utilize RNAi to protect their genomes against invading foreign genetic elements such as viruses. The formation of

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dsRNA during viral replication is interpreted by the cell as a signal for unwanted gene

activity (Ahlquist, P. *Science* 296, 1270-3 (2002), Plasterk, R.H. *Science* 296, 1263-5 (2002)). Dicer RNase III rapidly processes dsRNA to small dsRNA fragments of distinct size and structure, the small interfering RNAs (siRNAs), which direct the sequence-specific degradation of the single-stranded mRNAs of the invading genes 5 (Elbashir, S.M. et al. *Nature* 411, 494-8 (2001), Elbashir, S.M., et al. *Genes Dev* 15, 188-200 (2001), Hammond, S.M., et al. *Nature* 404, 293-6 (2000), Zamore, P.D., et al. *Cell* 101, 25-33 (2000)). Such siRNA duplexes have 2-3 nt 3′ overhanging ends and contain 5′ phosphate and free 3′ hydroxyl termini (Elbashir, S.M., et al. *Embo J* 20, 6877-88 (2001)). Cellular delivery of synthetic siRNA duplexes or introduction of 10 siRNA by plasmids or viral vectors is now widely used to disrupt the activity of cellular genes homologous in sequence to the introduced dsRNA.

An understanding of how siRNAs interact with mammalian systems is important for refining this gene silencing technology and for developing gene-specific therapeutic agents (Tuschl, T. et al. *Mol Interv* 2, 158-67 (2002)). For the recognition 15 of long dsRNA two different detection modes are known, the serine threonine kinase PKR (Williams, B.R. *Sci Signal Transduction Knowledge Enviroment* 89, RE2 (2001), Meurs, E.F. et al. *Virol* 66, 5805-14 (1992), Katze, M.G. et al. *Mol Cell Biol* 11, 5497-505 (1991)) and TLR3 (Alexopoulou, L., et al. *Nature* 413, 732-8 (2001)). While PKR is located in the cytosol, TLR3 is present in the endosomal compartment 20 (Matsumoto, M. et al. *J Immunol* 171, 3154-62 (2003)). TLR3 is a member of the Toll-like receptor family that has evolved to detect pathogen-specific molecules (Takeda, K., et al. *Annu Rev Immunol* 21, 335-76 (2003)).

PKR possesses two dsRNA-binding domains, one of which has high affinity for dsRNA, while the other shows considerably lower affinity. Full activation of the 25 PKR-mediated response requires simultaneous binding of dsRNA to both domains, which may be facilitated by long dsRNAs, e.g. dsRNAs exceeding 50-80 nucleotide pairs in length, and seems to require dimerization (Manche, L., et al., *Mol Cell Biol*, 12, 5238-48 (1992); Williams, B.G., *Oncogene*, 18, 6112-20 (1999)). High concentrations of dsRNAs, including dsRNAs of less than 50 nucleotide pairs, or of 30 other ligands for the dsRNA binding site (e.g. Alu RNAs) inhibit the activation of PKR. Early invenstigations seemed to prove that siRNA duplexes are short enough to bypass general dsRNA-induced unspecific effects in vertebrate cells (Bitko, V. et al.

BMC Microbiol, **1**, 34 (2001)). A number of more recent publications, however, indicates that a large array of genes is differentially regulated upon the introduction of short dsRNAs, including genes involved in the interferon pathway and specifically the activation of PKR, even if not to a similar extent as compared to the effect of long dsRNAs (Jackson, A.L. and Linsley, P.S., *Trends Genet*, **20**, 521-4 (2004); Jackson, A.L., et al., *Nat Biotechnol*, **21**, 635-7 (2003); Moss, E.G., and Taylor, J.M., *Nat Cell Biol*, **5**, 771-2 (2003); Bridge, A.J., et al., *Nat Genet*, **34**, 263-4 (2003); Sledz, C.A., et al., *Nat Cell Biol*, **5**, 834-9 (2003); Heidel, J.D., et al., *Nat Biotechnol*, **22**, 1579-81 (2004); Kim, D.H., et al., *Nat Biotechnol*, **22**, 321-5 (2004); Zheng, X., and Bevilacqua, P.C., *RNA*, **10**, 1934-45 (2004); Pebernard, S., and Iggo, R., *Differentiation*, **72**, 103–11 (2004)). Which genes are up- or downregulated seems to be at least partly siRNA-sequence specific, and the mechanism, or mechanisms, underlying this regulation remain(s) to be elucidated.

A second characteristic feature of viral nucleic acids used by the immune system to recognize viral infection are CpG motifs found in viral DNA, which are detected via TLR9 (Lund, J., et al. *J Exp Med* **198**, 513-520 (2003), Krug, A. et al. *Blood* **103**, 1433-7 (2004)). CpG motifs are unmethylated CG dinucleotides with certain flanking bases. The frequency of CpG motifs is suppressed in vertebrates, allowing the vertebrate immune system to detect microbial DNA based on such CpG motifs(Krieg, A.M. et al. *Nature* **374**, 546-9 (1995), Bauer, S. et al. *Proc Natl Acad Sci U S A* **98**, 9237-42 (2001), Wagner, H. *Curr Opin Microbiol* **5**, 62-9 (2002)). Like TLR3, TLR9 is located in the endosomal compartment where it directly binds to CpG motifs (Latz, E. et al. *Nat Immunol* **5**, 190-8 (2004)).

In addition to long dsRNA and CpG DNA, two recent publications suggest a third mechanism by which viral nucleic acids are recognized. These studies demonstrate that single-stranded RNA (ssRNA) of ssRNA viruses is detected via TLR7 (mouse and human) and TLR8 (only human) (Diebold, S.S., *Science* **303**, 1529-31 (2004), Heil, F. et al. *Science* **303**, 1526-9 (2004)). Guanine analogues have been identified earlier as specific ligands for TLR7 and TLR8 (Lee, J. et al. *Proc Natl Acad Sci U S A* **100**, 6646-51 (2003), Heil, F. et al. *Eur J Immunol* **33**, 2987-97 (2003)). Like TLR9 (receptor for CpG DNA)(Latz, E. et al. *Nat Immunol* **5**, 190-8 (2004)), TLR7 and TLR8 are located in the endosomal membrane.

Detection of viral nucleic acids leads to the production of type I IFN (IFN-α and IFN-β). The major producer of type I IFN in humans is the plasmacytoid dendritic cell (also called interferon producing cell, IPC). The plasmacytoid dendritic cell (PDC) is a highly specialized subset of dendritic cells that is thought to function as a sentinel for viral infection and that is responsible for the vast amount of type I IFN during viral infection (Asselin-Paturel, C. et al. *Nat Immunol* 2, 1144-50 (2001)). There is increasing evidence that PDC preferentially use nucleic acid-based molecular patterns to detect viral infection. TLR expression of human and mouse PDC is limited to TLR7 and TLR9 (Krug, A. et al. *Eur J Immunol* 31, 3026-37 (2001), Hornung, V. et al. *J Immunol* 168, 4531-7 (2002), Edwards, A.D. et al. *Eur J Immunol* 33, 827-33 (2003)).

Tokunaga et al, J. Natl. Cancer Inst. 72:955-962 (1984); Messina et al., J. Immunol. 147: 1759-1764 (1991); Krieg et al., Nature 374: 546-549 (1995); Sato et al, Science 273: 352-354 (1996), teach that the presence of CpG dinucleotides in certain sequence contexts in bacterial and synthetic oligodeoxyribonucleotides (CpG DNAs) are known to activate vertebrate innate immune reaction, T-cells and B cells.

Yamamoto et al., Jpn. J. Cancer Res. 79: 866-873 (1988); Halpern et al., Cell Immunol., 167: 72-78 (1996); Klinman et al., Proc. Natl. Acad. Sci. U.S.A. 93: 2879-2883 (1996); Zhao et al., Antisense Nucleic Acid Drug Dev. 7: 495-502 (1997) teach that the activation of immune cells by CpG DNA induces secretion of a number of cytokines, including IFN-.gamma., IL-12, TNF-.alpha., and IL-6, and stimulates expression of costimulatory surface molecules.

Krieg et al., supra; Yamamoto et al, J. Immunol. 148; 4072-4076 (1992); Tokunaga et al., Microbiol. Immunol. 36: 55-66 (1992); Liang et al., J. Clin. Invest. 98: 1119-1129 (1996); Hartmann et al., J. Immunol. 164: 1617-1624 (2000), teach that the presence of a CpG dinucleotide and the sequences flanking the dinucleotide play a critical role in determining the immunostimulatory activity of DNA, that CpG dinucleotides in palindromic or non-palindromic hexameric sequences ($P_1.\ P_2CGP_3P_4$) are required for immune stimulation, and further, that PuPuCGPyPy and PuTCG motifs optimally activate murine and human immune systems, respectively.

While these findings demonstrate that oligonucleotides are useful as immune stimulating agents, some problems with such use still exist. For example, long oligonucleotides are expensive to make and species specificity of flanking sequences limits the breadth of utility of any given oligonucleotide. There is, therefore, a need for less expensive immunostimulatory agents, and preferably immunostimulatory agents that have cross-species efficacy as well as a need to identify additional sequence specific motifs.

SUMMARY

The invention is based, at least in part, on the discovery that a particular sequence motif in a single or double stranded RNA molecule is effective at stimulating an immune response via IFN induction, particularly in cells expressing TLR7, such as plasmacytoid dendritic cells (PDC). Based on this discovery, the present invention provides immunostimulatory oligonucleotide agents that can be used to stimulate IFN production in a mammal as well as methods of selectively designing single stranded antisense agents and double stranded iRNA agents so as to induce a wanted immune response or to avoid inducing an unwanted immune response.

The invention provides therapeutic compositions comprising a single stranded or double stranded oligonucleotide as immunostimulatory agents as well as a method of using such composition for immunotherapy applications. The invention specifically provides methods and compositions for enhancing the immune response used for immunotherapy applications such as, but not limited to, treatment of cancer, autoimmune disorders, asthma, respiratory allergies, food allergies, and bacteria, parasitic, and viral infections in adult and pediatric human and veterinary applications. The invention further provides methods for making such compounds. In addition, compounds of the invention are useful as adjuvants in combination with DNA vaccines, antibodies, and allergens; and in combination with other immunostimulatory agents, chemotherapeutic agents, iRNA agents and/or antisense oligonucleotides.

The oligonucleotide agents of the present invention will comprise, consist of or consist essentially of the nucleotide sequence

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5'-GUCCUUCAA-3' (SEQ ID NO:1).

In other embodiments, the oligonucleotide will consist of, consist essentially of or comprise 4 or more, 5 or more, 6 or more, 7 or more or 8 or more contiguous nucleotides from SEQ ID NO:1, preferably taken from the 5' end of this sequence,

5   e.g. 5'-GUCC-3' (SEQ ID NO:2), 5'-GUCCU-3' (SEQ ID NO:3), 5'-GUCCUU-3' (SEQ ID NO:4), 5'-GUCCUUC-3' (SEQ ID NO:5), or 5'-GUCCUUCA-3' (SEQ ID NO:6).

Specifically, in one aspect, the invention provides a method of stimulating an immune response in a mammal comprising the step of administering to said mammal

10   an oligonucleotide agent consisting of, consisting essentially of, or comprising a sequence of 4 or more, 5 or more, 6 or more, 7 or more or 8 or more contiguous nucleotides from SEQ ID NO:1, preferably taken from the 5' end of this sequence. Said oligonucleotide agent may consist of, consist essentially of, or comprise SEQ ID NO:1, or a sequence that differs by not more than 1 or not more than 2 nucleotides

15   from SEQ ID NO:1. Said sequence may be chosen from the group of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6. The oligonucleotide may be an iRNA agent, or it may be a single stranded RNA agent.

In a second aspect, the invention provides a method of making an oligonucleotide agent so as to avoid stimulating an immune response in a mammal,

20   comprising the step of eliminating from a potential agent pool any agent that comprises a sequence of 4 or more, 5 or more, 6 or more, 7 or more or 8 or more contiguous nucleotides from SEQ ID NO:1, preferably taken from the 5' end of this sequence, or a sequence that differs from SEQ ID NO:1 by not more than one or not more than 2 nucleotides. The oligonucleotide may be an iRNA agent, or it may be a

25   single stranded RNA agent.

In a third aspect, the instant invention provides an isolated oligonucleotide agent consisting of, consisting essentially of, or comprising a sequence of 4 or more, 5 or more, 6 or more, 7 or more or 8 or more contiguous nucleotides from SEQ ID NO:1, preferably taken from the 5' end of this sequence. The oligonucleotide agent

30   may consist of, consist essentially of, or comprise SEQ ID NO:1, or a sequence that differs by not more than 1 or not more than 2 nucleotides from SEQ ID NO:1. Said

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sequence may be chosen from the group of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID
NO:4, SEQ ID NO:5, and SEQ ID NO:6. The oligonucleotide may be an iRNA agent,
or it may be a single stranded RNA agent. It may further comprise at least one 2'-
fluoro-modified nucleotide, wherein the 2'-fluoro-modified nucleotide is not part of a
5   sequence of 4 or more contiguous nucleotides from SEQ ID NO:1. Where the
oligonucleotide agent is an iRNA agent, it may be specific for (e.g. one strand is at
least partially complementary to) any one of the genes of Table 8.

In a fourth aspect, the present invention provides a method of making an
oligonucleotide agent so as to induce an immune response in a mammal, comprising
10 the step of adding to a potential agent pool any agent that comprises 4 or more, 5 or
more, 6 or more, 7 or more, or 8 or more contiguous nucleotides of SEQ ID NO:1, or
a sequence that differs from SEQ ID NO:1 by not more than one or not more than 2
nucleotides. The oligonucleotide may be an iRNA agent, or it may be a single
stranded RNA agent.

15   In a fith aspect, the invention provides a method of concomitantly inhibiting
the expression of a gene and inducing an immune response in a mammal, comprising
administering to said mammal an iRNA agent comprising a sequence of 4 or more, 5
or more, 6 or more, 7 or more, or 8 or more contiguous nucleotides of SEQ ID NO:1,
preferably taken from the 5' end of this sequence, or a sequence that differs from SEQ
20 ID NO:1 by not more than one or not more than 2 nucleotides. Said sequence may be
chosen from the group of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID
NO:5, and SEQ ID NO:6. Said gene may be any one of the genes of Table 8.

In a sixth aspect, the present invention provides a method of evaluating an
iRNA agent comprising:

25   providing a candidate iRNA agent;

reviewing the candidate iRNA agent to determine if it includes SEQ ID NO:1
or a sequence that differs from SEQ ID NO:1 by 1 or 2 nucleotides.

Said method may further comprise modifying the iRNA agent to remove the sequence of SEQ ID NO:1 or the sequence that differs from SEQ ID NO:1 by 1 or 2 nucleotides.

In a seventh aspect, the present invention provides a pharmaceutical composition comprising an oligonucleotide of the invention and a pharmaceutically acceptable carrier. Said pharmaceutical composition may be a vaccine.

In an eigth aspect, the present invention provides a method of making an oligonucleotide agent so as to avoid stimulating an immune response in a mammal, wherein said oligonucleotides comprises a sequence of 4 or more, 5 or more, 6 or more, 7 or more or 8 or more contiguous nucleotides from SEQ ID NO:1, comprising providing the oligonucleotide agent in such manner that it contains at least 2, or at least 4, 2'-*O*-methyl modified nucleotides. At least one, or at least two, of the 2'-*O*-methyl-modified nucleotides may be part of a sequence of 4 or more contiguous nucleotides from SEQ ID NO:1

BRIEF DESCRIPTION OF DRAWINGS

FIGs. 1A-1B: In HEK 293 cells siRNA can be used to inhibit TLR9 expression without inducing non-target-specific type I IFN-mediated STAT1- or STAT2-phosphorylating activity

HEK 293 cells (50.000/ well) were incubated with different RNA molecules and lipofectamine. A: HEK 293 cells expressing a construct of human TLR9 with a C-terminal YFP-tag were transfected with four siRNAs complementary to different regions of human TLR9 mRNA. Transfection with siRNA targeting GFP mRNA and TLR2 (referred to as sirna2.1 in the figures) mRNA served as a positive or negative control. At 20 hours the median fluorescence intensity of YFP-tag expression was analyzed by flow cytometry. Results are shown as mean values ± SEM (n=3). B: HEK 293 cells were incubated alone (lane 1), with lipofectamine (lane 2), with a 500 bp long dsRNA (10 μg/ml, lane 3), or were transfected with lipofectamine complexed with a 500 bp long dsRNA (lane 4), complexed with four different siRNAs targeting the human TLR9 mRNA (TLR9.1, TLR9.2, TLR9.3 and TLR9.4; lane 5 to 8; TLR9.1, TLR9.2, TLR9.3 and TLR9.4 are referred to as sirna9.1, sirna9.2, sirna9.3, and sirna9.4, respectively, in the figures). After 24 hours supernatants were harvested

and added to BL41 cells (1×10^6 cells/condition). Addition of recombinant IFN-β
(100 U/ml) served as a positive control (lane 9). After 30 min, BL41 cells were lysed
and subjected to SDS-page to examine STAT1- and STAT2-phosphorylation.
Supernatants were collected from quadruplicates. One representative experiment out

5   of three is shown.

FIGs. 2A-2E: Sequence-dependent induction of IFN-α in plasmacytoid dendritic cells by siRNA is based on motif recognition on the single-strand level

PDC (50.000/ well) were transfected with different RNA oligonucleotides
using lipofectamine (0.5 μl). After 36 hours, IFN-α production was measured in the

10   supernatants. A: PDC were transfected with 200 ng of four different siRNAs
(TLR9.1, TLR9.2, TLR9.3 and TLR9.4). The TLR9-ligand CpG-A (ODN 2216, grey
bar) served as a positive control for IFN-α induction. Results represent the mean IFN-
α production ± SEM from eleven individual donors ($p < 0,0001$ for TLR9.1 vs.
TLR9.2, $p < 0,0001$ for TLR9.1 vs. TLR9.3, $p < 0,0001$ for TLR9.3 vs. TLR9.1 and p

15   $< 0,001$ for TLR9.3 vs. TLR9.4). B: PDC were transfected with decreasing amounts
(200 ng, 100 ng, 50 ng, 25 ng) of TLR9.1, TLR9.2, TLR9.3 or TLR9.4. Results
represent the mean IFN-α production ± SEM from three individual donors. C: PDC
were transfected with either siRNA9.2 duplex or the corresponding sense and anti-
sense strands. Results from ten individual donors were summarized and are depicted

20   as mean values ± SEM. D (left panel): The FITC-modified version of the siRNA9.2
sense strand or the corresponding annealed siRNA-duplex were incubated with or
without RNAse at 266 μg/ml. After three hours 4 μl of this preparation were analyzed
on a 3 % agarose-gel, whereas untreated samples were included as controls. D (right
panel): Subsequently samples were purified via siRNA-purification-columns to

25   remove RNase and 3μl of this preparation was transfected into PDCs. After 36 h IFN-
α -production was assessed by ELISA. Data from one of two representative
experiments are depicted. E: The self-stabilizing single-stranded hairpin-version of
siRNA-duplex 9.2 (see table 2) is compared to the siRNA9.2 duplex. Data from two
independent experiments are presented as means ± SEM.

FIGs. 3A-3B: A sequence of 9 bases at the 3'end of the RNA9.2 sense strand is
responsible for the immunostimulatory activity

    PDC were transfected with different RNA oligonucleotides. After 36 hours,
IFN-α production was measured in the supernatant. A: PDC were transfected with the

5   sense strand of TLR9.2 (RNA9.2s, the original sequence designated "n") and with
fluorescein-tagged (5'F or 3'F) versions of RNA9.2s. * indicate $p<0.05$. B: PDC were
transfected with the sense strand of TLR9.2, a 19-mer polyA-oligonucleotide, a panel
of RNA9.2s derivatives in which base number 8 to 12 counting from the 5'end were
replaced by adenosine (L8A to L12A), a panel of RNA9.2s derivatives in which one,

10   two, three or six bases within the 9 bases counting from the 3'end (putative motif)
were replaced by adenosine or uridine (in order to disrupt the putative 9mer motif), or
two shortened versions of RNA9.2s (a 16mer and a 12mer both containing the
putative 9mer motif of the 3'end of RNA9.2s) and a 19mer RNA oligonucleotide
containing the putative 9mer motif two times within its sequence (DR). A detailed list

15   of all sequences used is provided in Table 2. Results from four individual donors are
depicted as mean values ± SEM.

FIGs. 4A-4B: LNA-modifications of the sense and the anti-sense strand in siRNA
TLR9.2 reveal IFN-α induction and silencing as two independent activities of siRNA

    PDC were transfected with different RNA oligonucleotides. After 36 hours,

20   IFN-α production was measured in the supernatant. A: The sense strand of TLR9.2
(RNA9.2s) and its derivatives with and without LNA-modification of the 5'and the
3'end (n, 5'LNA, 3'LNA, 5'3'LNA) are compared (see Table 2). B: Different LNA-
modified versions of the sense and the antisense strand of siRNA9.2 were annealed to
duplexes and transfected into PDC. After 36 hours, IFN-α production was measured

25   in the supernatants (black bars). Results from five individual donors are depicted as
mean values ± SEM. In addition, HEK 293 cells stably expressing a construct of
human TLR9 with a C-terminal YFP-tag were transfected with the same type of
siRNA9.2 derivatives. Transfection with siRNA-duplexes targeting GFP, TLR2 or
TLR4 mRNA served as controls. 20 hours after transfection the median fluorescence

30   intensity of YFP-tag expression was analyzed by flow cytometry (open bars). Data are
depicted as percentage of TLR9-expression referring to siRNA2.1 as 100 % and the
unmodified siRNA9.2 as 0 %. Results are shown as mean values ± SEM (n=3).

FIGs. 5A-5D: Sequence dependent induction of systemic immune responses by siRNA in mice *in vivo.*

A: Murine bone-marrow cultures (129Sv) were stimulated with siRNA TLR9.2 duplex, sense or anti-sense strand or a 19mer poly(A)-oligonucleotide

5   complexed to polycationic peptide. ODN 1826 (6 µg/ml) and R848 (1H-Imidazo(4,5-c)quinoline-1-ethanol(ethoxymethyl)-Alpha; 0,5 µg/ml) served as positive controls. After 36 hours, supernatants were collected and IFN-α production was assessed by ELISA. Data are presented as mean values ± SEM from triplicates of pooled bone-marrow cultures from three individual mice. B: 5 µg of siRNA TLR2.1, TLR9.2, or

10   single strands TLR9.2a (sense strand) or TLR9.2as (anti-sense strand) were complexed with DOTAP and administered via i.v. injection to 129Sv mice (3 mice per group). DOTAP without RNA was included as a control. After seven hours serum was collected and IFN-α production was assessed by ELISA. Data are presented as mean values ± SEM. C: 41 hours after injection, spleen cells were isolated and CD8+

15   T cells and MDCs were identified in spleen cells by using anti-CD3, anti-CD8, anti-CD11b and anti-CD11c antibodies. CD69 or CD86-expression was assessed using an appropriate isotype control Ab. Results from three mice per group are presented as mean values ± SEM. D: The siRNA TLR9.2, CpG ODN 1826 (both 5 µg) or HBS with or without DOTAP-complexation were administered via i.v. injection to

20   129P2/OlaHsd mice (3 mice per group). After eighteen hours, serum was collected and IFN-α production was assessed by ELISA (left panel). In addition, CD86 expression was analysed on splenic MDCs 41 hours after injection (right panel). Data are presented as mean values ± SEM.

FIGs. 6A-6C: TLR7 is required for siRNA recognition

25   A: Murine bone-marrow cultures from wild-type (black bars) or TLR7-/- (open bars) C57BL/6 mice were incubated with siRNA TLR9.2, with the sense strand or the antisense strand of TLR9.2 or a 19mer poly(A) oligonucleotide complexed with polycationic peptide. CpG ODN 1826 (TLR9) and loxoribine (TLR7) were used at a concentration of 6 µg/ml or 0,5 µg/ml respectively. Supernatants were collected 36 h

30   after stimulation and IFN-α -production was assessed by ELISA. Data are presented as mean values ± SEM from triplicates of pooled bone-marrow cultures from two individual mice. B: 5 µg of siRNA TLR9.2 was complexed with DOTAP and injected

i.v. into either wildtype (black bars) or TLR7 -/-(open bars) C57BL/6 mice . Two
mice per group were used. After 7 and 18 hours serum was collected and IFN-α
production was assessed by ELISA. C: 41 hours after injection, spleen cells were
isolated. CD8+ and CD4+ T cells were identified in spleen cells by using anti-CD3

5   and anti-CD8 antibodies. CD69-expression was assessed using an appropriate isotype
control antibody (grey bar). One representative histogram is shown for CD8+ T cells.
The expression of CD69 or CD86 was analyzed on PDC (CD11c$^+$, CD11b$^-$ and
B220^{++}) and myeloid dendritic cells (MDC: CD11c^{++}, CD11b^{++} and B220$^-$).
Expression of CD69 and CD86 is presented as means ± SEM.

10   **DETAILED DESCRIPTION**

       The invention is based, at least in part, on the discovery that a particular
sequence motif in a single or double stranded RNA molecule is effective at
stimulating an immune response via IFN induction, particularly in cells expressing
TLR7, such as plasmacytoid dendritic cells (PDC). Based on this discovery, the

15   present invention provides immunostimulatory oligonucleotide agents that can be
used to stimulate IFN production in a mammal as well as methods of selectively
designing single stranded antisense agents and double stranded iRNA agents so as to
induce a wanted immune response or to avoid inducing an unwanted immune
response.

20   Specifically, the present invention provides therapeutic compositions
comprising a single stranded or double stranded oligonucleotide as an
immunostimulatory agent as well as a method of using such composition for
immunotherapy applications. The invention specifically provides methods and
compositions for enhancing the immune response used for immunotherapy

25   applications such as, but not limited to, treatment of cancer, autoimmune disorders,
asthma, respiratory allergies, food allergies, and bacteria, parasitic, and viral
infections in adult and pediatric human and veterinary applications. The invention
further provides methods for making such compounds. In addition, compounds of the
invention are useful as adjuvants in combination with DNA vaccines, antibodies, and

30   allergens; and in combination with other immunostimulatory agents,
chemotherapeutic agents, iRNA agents and/or antisense oligonucleotides.

                                        12

The present invention provides oligonucleotide agents that comprise, consist of or consist essentially of the nucleotide sequence

5'-GUCCUUCAA-3' (SEQ ID NO:1).

In other embodiments, the oligonucleotide agent will consist of, consist essentially of or comprise 4 or more, 5 or more, 6 or more, 7 or more or 8 or more contiguous nucleotides from SEQ ID NO:1, preferably taken from the 5' end of this sequence, e.g. 5'-GUCC-3' (SEQ ID NO:2), 5'-GUCCU-3' (SEQ ID NO:3), 5'-GUCCUU-3' (SEQ ID NO:4), 5'-GUCCUUC-3' (SEQ ID NO:5), or 5'-GUCCUUCA-3' (SEQ ID NO:6).

As used herein, an oligonucleotide agent consists of SEQ ID NO:1 when it does not contain other nucleotides in the agent. As used herein, an oligonucleotide agent consists essentially of SEQ ID NO:1 when it contains no more than 1, 2, 3, or 4 other nucleotides in the agent. As used herein, an oligonucleotide agent comprises SEQ ID NO:1 when it contains other nucleotides in the agent. Preferably, such agents will contain no more than 21 other nucleotides and more preferably no more than from about 15 to about 10 other nucleotides in the agent where the agent does not comprise a double stranded structure. Oligonucleotide agents comprising a double stranded structure, e.g. iRNA agents, will contain no more than 21, 15 or 10 other nucleotides in a strand comprising SEQ ID NO:1, and preferably no more than 30, 24, or 19 nucleotides in a strand not comprising SEQ ID NO:1.

As used herein, the term "oligonucleotide" refers to a polynucleotide formed from a plurality of linked nucleoside units. Such oligonucleotides can be obtained from existing nucleic acid sources, including genomic or cDNA, but are preferably produced by synthetic methods. In preferred embodiments each nucleoside unit includes a heterocyclic base and a pentofuranosyl, trehalose, arabinose, 2'-deoxy-2'-substituted arabinose, 2'-O-substituted arabinose or hexose sugar group. The nucleoside residues can be coupled to each other by any of the numerous known internucleoside linkages. Such internucleoside linkages include, without limitation, phosphodiester, phosphorothioate, phosphorodithioate, alkylphosphonate, alkylphosphonothioate, phosphotriester, phosphoramidate, siloxane, carbonate, carboalkoxy, acetamidate, carbamate, morpholino, borano, thioether, bridged

phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate, and sulfone internucleoside linkages. The term "oligonucleotide" also encompasses polynucleosides having one or more stereospecific internucleoside linkage (e.g., (R_p)- or (S_p)-phosphorothioate, alkylphosphonate, or phosphotriester linkages).

5   The oligonucleotides of the invention can include naturally occurring nucleosides, modified nucleosides, or mixtures thereof, so long as they consist of, consist essentially of or comprise SEQ ID NO:1. As used herein, the term "modified nucleoside" is a nucleoside that includes a modified heterocyclic base, a modified sugar moiety, or a combination thereof. In some embodiments, the modified

10   nucleoside is a non-natural pyrimidine or purine nucleoside, as herein described. In some embodiments, the modified nucleoside is a 2'-substituted ribonucleoside an arabinonucleoside or a 2'-deoxy-2'-substituted-arabinoside.

        As used herein, the term "2'-substituted ribonucleoside" or "2'-substituted arabinoside" includes ribonucleosides or arabinonucleoside in which the hydroxyl

15   group at the 2' position of the pentose moiety is substituted to produce a 2'-substituted or 2'-O-substituted ribonucleoside. Preferably, such substitution is with a lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an aryl group having 6-10 carbon atoms, wherein such alkyl, or aryl group may be unsubstituted or may be substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl,

20   acyloxy, alkoxy, carboxyl, carboalkoxy, or amino groups. Examples of 2'-O-substituted ribonucleosides or 2'-O-substituted-arabinosides include, without limitation 2'-O-methylribonucleosides or 2'-O-methylarabinosides and 2'-O-methoxyethylribonucleosides or 2'-O-methoxyethylarabinosides.

        The term "2'-substituted ribonucleoside" or "2'-substituted arabinoside" also

25   includes ribonucleosides or arabinonucleosides in which the 2'-hydroxyl group is replaced with a lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an amino or halo group. Examples of such 2'-substituted ribonucleosides or 2'-substituted arabinosides include, without limitation, 2'-amino, 2'-fluoro, 2'-allyl, and 2'-propargyl ribonucleosides or arabinosides.

30   The term "oligonucleotide" includes hybrid and chimeric oligonucleotides. A "chimeric oligonucleotide" is an oligonucleotide having more than one type of

internucleoside linkage. One preferred example of such a chimeric oligonucleotide is a chimeric oligonucleotide comprising a phosphorothioate, phosphodiester or phosphorodithioate region and non-ionic linkages such as alkylphosphonate or alkylphosphonothioate linkages (see e.g., Pederson et al. U.S. Pat. Nos. 5,635,377 and 5,366,878).

A "hybrid oligonucleotide" is an oligonucleotide having more than one type of nucleoside. One preferred example of such a hybrid oligonucleotide comprises a ribonucleotide or 2'-substituted ribonucleotide region, and a deoxyribonucleotide region (see, e.g., Metelev and Agrawal, U.S. Pat. Nos. 5,652,355, 6,346,614 and 6,143,881).

An "immunostimulatory" agent, or an agent that "stimulates an immune response" herein means an agent that stimulates in a cell *in vitro* or in an organism *in vivo* a response that is commonly understood to be part of the natural defenses of an organism against biological pathogens, e.g. the immune system. Such response can be, for example, without limitation, be the production of antibodies or cytokines, e.g. interferons, e.g. interferon alpha (IFN-α).

In another embodiment, the invention provides immunomodulatory oligonucleotide conjugates and oligonucleotide agent conjugates, for example comprising an immunomodulatory oligonucleotide or an oligonucleotide agent, as described above, and an antigen conjugated to the oligonucleotide agent at a position other than the accessible 5' end. In some embodiments, the non-nucleotidic linker comprises an antigen, which is conjugated to the oligonucleotide. In some other embodiments, the antigen is conjugated to the oligonucleotide at a position other than its 3' end. In some embodiments, the antigen produces a vaccine effect. Other oligonucleotide agent conjugates are further described below.

Where an oligonucleotide agent is conjugated to an antigen, the antigen is preferably selected from the group consisting of antigens associated with a pathogen, antigens associated with a cancer, antigens associated with an auto-immune disorder, and antigens associated with other diseases such as, but not limited to, veterinary or pediatric diseases. As used herein, the term "associated with" means that the antigen is present when the pathogen, cancer, auto-immune disorder, food allergy, respiratory

15

allergy, asthma or other disease is present, but either is not present, or is present in reduced amounts, when the pathogen, cancer, auto-immune disorder, food allergy, respiratory allergy, or disease is absent.

The immunomodulatory oligonucleotide or oligonucleotide agent is covalently linked to the antigen, or it is otherwise operatively associated with the antigen. As used herein, the term "operatively associated with" refers to any association that maintains the activity of both oligonucleotide agent and antigen. Nonlimiting examples of such operative associations include being part of the same liposome or other such delivery vehicle or reagent. In embodiments wherein the oligonucleotide agent is covalently linked to the antigen, such covalent linkage preferably is at any position on the oligonucleotide agent other than an accessible 5' end of an immunostimulatory oligonucleotide. For example, the antigen may be attached at an internucleoside linkage or may be attached to the non-nucleotidic linker. Alternatively, the antigen may itself be the non-nucleotidic linker.

In a preferred embodiment, the oligonucleotide or oligonucleotide agent is an iRNA agent, such as an antisense agent or siRNA agent. In that sense, oligonucleotide or oligonucleotide agent, as used herein, can also refer to a complex consisting of more than one, and preferably two, oligonucleotide molecules, which occur essentially only in direct association, e.g. by hybridizing to each other, under certain conditions, such as those found in the serum of mammals, e.g. humans, or in the cytoplasm of mammalian, and particularly human, cells. To be an oligonucleotide agent hereunder, at least one of the more than one oligonucleotide molecules forming the complex consists, consists essentially of, or comprises SEQ ID NO:1.

An iRNA agent, as used herein, is an agent capable of specifically interfering with the expression of a target gene, such as an antisense or siRNA agent. Typically, an iRNA agent comprises an oligonucleotide sequence which is complementary to a part of an mRNA encoded by the target gene. It interferes with the expression of the target gene by any mechanism, e.g. by blocking the translation of the mRNA, by initiating the degradation of the mRNA, e.g. via an RNA interference mechanism, or by blocking the transcription of the gene, e.g. via DNA methylation.

iRNA Agent Design

The present invention further provides methods of designing/selecting an
iRNA agent, such as an antisense or siRNA agent, such that it will either induce an
inflammatory response or avoid inducing an inflammatory response. Specifically, this
method comprises the step of either including SEQ ID NO:1, or a fragment thereof in
an oligonucleotide agent, such as an antisense or siRNA agent, when an
IFN/inflammatory response is wanted or not including this sequence in the agent
when an inflammatory response is not wanted.

Accordingly, the present invention provides, *inter alia*, iRNA agents
comprising an antisense strand and, optionally, a sense strand, comprising a sequence
of at least 15, 16, 17, 18, 19, 20, 21 or 23 nucleotides, wherein at least one of the
sequences of the antisense and the optional sense strand comprises SEQ ID NO:1
when IFN production is wanted and wherein none of the antisense strand and the
optional sense strand will contain this sequence when IFN production is to be
avoided..

The antisense strand of an iRNA agent should be equal to or at least, 15, 16
17, 18, 19, 25, 29, 40, or 50 nucleotides in length. It should be equal to or less than
50, 40, or 30, nucleotides in length. Preferred ranges are 15-30, 17 to 25, 19 to 23, and
19 to 21 nucleotides in length. .

The sense strand, if any, of an iRNA agent should be equal to or at least 15, 16
17, 18, 19, 25, 29, 40, or 50 nucleotides in length. It should be equal to or less than
50, 40, or 30 nucleotides in length. Preferred ranges are 15-30, 17 to 25, 19 to 23, and
19 to 21 nucleotides in length.

The double stranded portion, if any, of an iRNA agent should be equal to or at
least, 15, 16 17, 18, 19, 20, 21, 22, 23, 24, 25, 29, 40, or 50 nucleotide pairs in length.
It should be equal to or less than 50, 40, or 30 nucleotides pairs in length. Preferred
ranges are 15-30, 17 to 25, 19 to 23, and 19 to 21 nucleotides pairs in length.

In designing iRNA agents according to the invention, it may be advantageous
to first identify a region in an mRNA sequence of a target gene which is either

17

complementary (for use in antisense oligonucleotides and siRNA antisense strands) or
identical (for use in an siRNA sense strand) to 4 or more, 5 or more, 6 or more, 7 or
more or 8 or more contiguous nucleotides from SEQ ID NO:1. The iRNA agent can
then be chosen to comprise the sequence complementary or identical sequence to this

5   region, plus a number of suitable additional nucleotides to confer target gene
expression inhibitory activity. Therein, SEQ ID NO:1 may be comprised anywhere
within the iRNA agent, e.g. in a 3'- or 5'-terminal region, or anywhere within, of
either a sense or antisense strand of the iRNA agent.

However, where it is desired to inhibit a certain gene where its mRNA does

10   not comprise a region of complete complementarity or identity to SEQ ID NO:1, an
iRNA agent comprising mismatches to the target gene mRNA may also be used.
Since mismatches are most tolerated in the terminal regions of either strand of, for
example, an siRNA, the mismatches will best be introduced in these terminal regions.
For example, the 3'-most or 5'-most 4, 5, 6, 7, 8 or 9 nucleotides of the sense strand or

15   the 3'-most 4, 5, 6, 7, 8 or 9 nucleotides of the antisense strand of an siRNA may be
chosen from SEQ ID NO:1, wherein not more than 1, not more than 2, or not more
than 3 nucleotides represent a mismatch to the target mRNA, and the remaining
nucleotides are chosen fully identical or complementary to the target mRNA. The
nucleotides in positions 2 - 9 (counting 5' → 3') of the antisense strand, however, are

20   believed to be critical for target mRNA recognition (Haley, B., and Zamore, P.D., *Nat
Struct Mol Biol*, 11, 599-606 (2004)). Therefore, it is preferred that in this region
(sometimes referred to as the "seed region") there is perfect complementarity to the
target mRNA.

In such embodiment, said target gene can be essentially any gene the sequence

25   of which enables the design of a fully matched or partly mismatched iRNA agent as
described above. The gene may, for example be a mammalian gene, *e.g.* a human
gene. For example, without limitation, such a gene may be an oncogene, a gene
involved in an immune response, a gene involved in metabolism, or a gene encoding a
growth factor, a transcription factor, or a receptor. Table 7 contains a non-limiting list

30   of names of exemplary gene transcripts, as obtained from BLAST searching databases
of human mRNA sequences, which may be inhibited in this fashion, as they comprise
a sequence identical or complementary to SEQ ID NO:1. Alternatively, the target

gene may be a gene from an organism that is pathogenic to animals, pereferably mammals, more preferably humans, such as a bacterium or a virus.

iRNA agent Chemistry

iRNA agents discussed herein include otherwise unmodified RNA as well as RNA which have been modified, *e.g.*, to improve efficacy, and polymers of nucleoside surrogates. Unmodified RNA refers to a molecule in which the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are the same or essentially the same as that which occur in nature, preferably as occur naturally in the human body. The art has referred to rare or unusual, but naturally occurring, RNAs as modified RNAs, see, *e.g.*, Limbach *et al.*, (1994) *Nucleic Acids Res.* 22: 2183-2196. Such rare or unusual RNAs, often termed modified RNAs (apparently because these are typically the result of a post-transcriptional modification) are within the term unmodified RNA, as used herein. Modified RNA as used herein refers to a molecule in which one or more of the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are different from that which occurs in nature, preferably different from that which occurs in the human body. While they are referred to as modified "RNAs," they will of course, because of the modification, include molecules which are not RNAs. Nucleoside surrogates are molecules in which the ribophosphate backbone is replaced with a non-ribophosphate construct that allows the bases to the presented in the correct spatial relationship such that hybridization is substantially similar to what is seen with a ribophosphate backbone, *e.g.*, non-charged mimics of the ribophosphate backbone. Examples of each of the above are discussed herein.

Enhanced Nuclease Resistance

For increased nuclease resistance and/or binding affinity to a target mRNA, an oligonucleotide agent can include, for example, 2'-modified ribose units and/or phosphorothioate linkages. *E.g.*, the 2' hydroxyl group (OH) can be modified or replaced with a number of different "oxy" or "deoxy" substituents.

Examples of "oxy"-2' hydroxyl group modifications include alkoxy or aryloxy (OR, *e.g.*, R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG), $O(CH_2CH_2O)_nCH_2CH_2OR$; "locked" nucleic acids (LNA)

19

in which the 2' hydroxyl is connected, e.g., by a methylene bridge, to the 4' carbon of the same ribose sugar; O-AMINE and aminoalkoxy, $O(CH_2)_nAMINE$, (e.g., AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl amino, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino). It is

5   noteworthy that oligonucleotides containing only the methoxyethyl group (MOE), ($OCH_2CH_2OCH_3$, a PEG derivative), exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

"Deoxy" modifications include hydrogen (i.e. deoxyribose sugars, which are of particular relevance to the overhang portions of partially ds RNA); halo (e.g.,

10   fluoro); amino (e.g. NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); $NH(CH_2CH_2NH)_nCH_2CH_2-AMINE$ (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl amino, arylamino, diaryl amino, heteroaryl amino,or diheteroaryl amino), $-NHC(O)R$ (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano;

15   mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with e.g., an amino functionality.

Preferred substitutents are 2'-methoxyethyl, 2'-OCH_3, 2'-O-allyl, 2'-C- allyl, and 2'-fluoro.

To maximize nuclease resistance, the 2' modifications can be used in

20   combination with one or more phosphate linker modifications (e.g., phosphorothioate).

The inclusion of furanose sugars in the oligonucleotide backbone can also decrease endonucleolytic cleavage. An oligonucleotide agent can be further modified by including a 3' cationic group, or by inverting the nucleoside at the 3'-terminus with

25   a 3'-3' linkage. In another alternative, the 3'-terminus can be blocked with an aminoalkyl group, e.g., a 3' C5-aminoalkyl dT. Other 3' conjugates can inhibit 3'-5' exonucleolytic cleavage. While not being bound by theory, a 3' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 3'-end of oligonucleotide. Even small alkyl chains,

aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

Similarly, 5' conjugates can inhibit 5'-3' exonucleolytic cleavage. While not being bound by theory, a 5' conjugate, such as naproxen or ibuprofen, may inhibit exonucleolytic cleavage by sterically blocking the exonuclease from binding to the 5'-end of oligonucleotide. Even small alkyl chains, aryl groups, or heterocyclic conjugates or modified sugars (D-ribose, deoxyribose, glucose etc.) can block 3'-5'-exonucleases.

Tethered Ligands

The properties of an oligonucleotide agent, including its pharmacological properties, can be influenced and tailored, for example, by the introduction of ligands, e.g. tethered ligands. An oligonucleotide agent comprising a tethered ligand may also be referred to herein as a conjugate or bioconjugate.

A wide variety of entities, e.g., ligands, can be tethered to an oligonucleotide agent, e.g., to the carrier of a ligand-conjugated monomer subunit. Examples are described below in the context of a ligand-conjugated monomer subunit but that is only preferred, entities can be coupled at other points to an oligonucleotide agent.

Preferred moieties are ligands, which are coupled, preferably covalently, either directly or indirectly via an intervening tether, to the carrier. In preferred embodiments, the ligand is attached to the carrier *via* an intervening tether. The ligand or tethered ligand may be present on the ligand-conjugated monomer when the ligand-conjugated monomer is incorporated into the growing strand. In some embodiments, the ligand may be incorporated into a "precursor" ligand-conjugated monomer subunit after a "precursor" ligand-conjugated monomer subunit has been incorporated into the growing strand. For example, a monomer having, e.g., an amino-terminated tether, e.g., TAP-$(CH_2)_nNH_2$ may be incorporated into a growing sense or antisense strand. In a subsequent operation, i.e., after incorporation of the precursor monomer subunit into the strand, a ligand having an electrophilic group, e.g., a pentafluorophenyl ester or aldehyde group, can subsequently be attached to the precursor ligand-conjugated

monomer by coupling the electrophilic group of the ligand with the terminal
nucleophilic group of the precursor ligand-conjugated monomer subunit tether.

In preferred embodiments, a ligand alters the distribution, targeting or lifetime
of an oligonucleotide agent into which it is incorporated. In preferred embodiments a
5   ligand provides an enhanced affinity for a selected target, e.g., molecule, cell or cell
type, compartment, e.g., a cellular or organ compartment, tissue, organ or region of
the body, as, e.g., compared to a species absent such a ligand.

Preferred ligands can improve transport, hybridization, and specificity
properties and may also improve nuclease resistance of the resultant natural or
10   modified oligoribonucleotide, or a polymeric molecule comprising any combination
of monomers described herein and/or natural or modified ribonucleotides.

Ligands in general can include therapeutic modifiers, e.g., for enhancing
uptake; diagnostic compounds or reporter groups e.g., for monitoring distribution;
cross-linking agents; nuclease-resistance conferring moieties; and natural or unusual
15   nucleobases. General examples include lipophilic moleculeses, lipids, lectins, steroids
(e.g.,uvaol, hecigenin, diosgenin), terpenes (e.g., triterpenes, e.g., sarsasapogenin,
Friedelin, epifriedelanol derivatized lithocholic acid), vitamins, carbohydrates(e.g., a
dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid), proteins,
protein binding agents, integrin targeting molecules,polycationics, peptides,
20   polyamines, and peptide mimics.

The ligand may be a naturally occurring or recombinant or synthetic molecule,
such as a synthetic polymer, e.g., a synthetic polyamino acid. Examples of polyamino
acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-
glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied)
25   copolymer, divinyl ether-maleic anhydride copolymer, N-(2-
hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG),
polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-
isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include:
polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine,
30   pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine,

22

arginine, amidine, protamine, cationic moieties, e.g., cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

Ligands can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a thyrotropin, melanotropin, surfactant protein A, Mucin carbohydrate, a glycosylated polyaminoacid, transferrin, bisphosphonate, polyglutamate, polyaspartate, or an RGD peptide or RGD peptide mimetic.

Ligands can be proteins, e.g., glycoproteins, lipoproteins, e.g. low density lipoprotein (LDL), or albumins, e.g. human serum albumin (HSA), or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-glucosamine, multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB.

The ligand can be a substance, e.g., a drug, which can increase the uptake of the oligonucleotide agent into the cell, for example, by disrupting the cell's cytoskeleton, e.g., by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

In one embodiment, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, e.g., human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, e.g., liver tissue, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, e.g., HSA.

A lipid based ligand can be used to modulate, e.g., control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In another embodiment, the ligand is a moiety, e.g., a vitamin or nutrient, which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include the B vitamins, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells.

In another embodiment, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennapedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

Preferred ligands confer to the agent the ability to bind to a cell, preferably to a cell of a specific cell type most relevant to the disease state in question. For example, the asialoglycoprotein receptor (ASGPr) (Wu and Wu, 1987, J. Biol. Chem. 262, 4429-4432) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as asialoorosomucoid (ASOR). In another example, the folate receptor is overexpressed in many cancer cells. Binding of such glycoproteins, synthetic glycoconjugates, or folates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example, triatennary structures are bound with greater affinity than biatenarry or monoatennary chains (Baenziger and Fiete, 1980, Cell, 22, 611-620; Connolly et al., 1982, J. Biol. Chem., 257, 939-945). Lee and Lee, 1987, Glycoconjugate J., 4, 317-328, obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to

24

galactose. This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates (Ponpipom et al., 1981, J. Med. Chem., 24, 1388-1395). The use of galactose, galactosamine, or folate based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to, for example, the treatment of liver disease, cancers of the liver, or other cancers. The use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds required for treatment. Furthermore, therapeutic bioavailability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of nucleic acid bioconjugates of the invention. Non-limiting examples of such bioconjugates are described in Vargeese et al., U.S. Ser. No. 10/201,394, filed Aug. 13, 2001; and Matulic-Adamic et al., U.S. Ser. No. 60/362,016, filed Mar. 6, 2002.

5'-Phosphate Modifications

In other embodiments, oligonucleotide agents are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications of the antisense strand include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)2(O)P-O-5'); 5'-diphosphate ((HO)2(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)2(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure. Other suitable 5'-phosphate modifications will be known to the skilled person.

Formulation

The oligonucleotide agents described herein can be formulated for administration to a subject, preferably for systemic administration, e.g. parenteral or oral administration, including, without limitation, intravenous, intramuscular, intraperitoneal, rectal, intradermal, subcutaneous, or percutaneous administration, or for the targeted delivery to tissues, e.g. the lungs and nasal passage (respiratory tissues), e.g. via inhalation or intranasal administration, the liver, kidney, spleen, brain, spinal cord, eye, skin, gut, mucosa, placenta, or any other tissue or organ that is a preferred target for the effect of the oligonucleotide agent.

For ease of exposition, the formulations, compositions, and methods in this section are discussed largely with regard to unmodified oligonucleotide agents. It should be understood, however, that these formulations, compositions, and methods can be practiced with other oligonucleotide agents, e.g., modified oligonucleotide agents, and such practice is within the invention.

A formulated oligonucleotide agent composition can assume a variety of states. In some examples, the composition is at least partially crystalline, uniformly crystalline, and/or anhydrous (e.g., less than 80, 50, 30, 20, or 10% water). In another example, the oligonucleotide agent is in an aqueous phase, e.g., in a solution that includes water, this form being the preferred form for parenteral administration.

The aqueous phase or the crystalline compositions can be incorporated into a delivery vehicle, e.g., a liposome (particularly for the aqueous phase), or a particle (e.g., a microparticle as can be appropriate for a crystalline composition). Generally, the oligonucleotide agent composition is formulated in a manner that is compatible with the intended method of administration.

An oligonucleotide agent preparation can be formulated in combination with another agent, e.g., another therapeutic agent or an agent that stabilizes an oligonucleotide agent, e.g., a protein that complexes with the oligonucleotide agent. Still other agents include chelators, e.g., EDTA (e.g., to remove divalent cations such as Mg^{2+}), salts, nuclease inhibitors (e.g., a broad specificity nuclease inhibitor such as RNAsin) and so forth.

In one embodiment, the oligonucleotide agent preparation includes another oligonucleotide agent, e.g., an siRNA agent that can mediate RNAi with respect to a target gene. In such a use, a target gene is disrupted in a cell as well as the cell being stimulated to produce IFN using the oligonucleotide agent of the present invention. Such cotherapy is important in treating disorders such as cancers, and viral infections.

Methods for the delivery of nucleic acid molecules are described in Akhtar et al., 1992, Trends Cell Bio., 2, 139; Delivery Strategies for Antisense Oligonucleotide Therapeutics, ed. Akhtar, 1995, Maurer et al., 1999, Mol. Membr. Biol., 16, 129-140; Hofland and Huang, 1999, Handb. Exp. Pharmacol., 137, 165-192; and Lee et al.,

2000, ACS Symp. Ser., 752, 184-192, all of which are incorporated herein by reference. Beigelman et al., U.S. Pat. No. 6,395,713 and Sullivan et al., PCT WO 94/02595 further describe the general methods for delivery of nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule.

5 Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as biodegradable polymers, hydrogels, cyclodextrins (see for example Gonzalez et al., 1999, Bioconjugate Chem., 10, 1068-1074; Wang et al., International PCT publication Nos.

10 WO 03/47518 and WO 03/46185), poly(lactic-co-glycolic)acid (PLGA) and PLCA microspheres (see for example U.S. Pat. No. 6,447,796 and U.S. Patent Application Publication No. U.S. 2002130430), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). Alternatively, the nucleic acid/vehicle combination is

15 locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described in Conry et al., 1999, Clin. Cancer Res., 5, 2330-2337 and Barry et al., International PCT Publication No. WO 99/31262.

20 In another embodiment, the nucleic acid molecules of the invention can also be formulated or complexed with polyethyleneimine and derivatives thereof, such as polyethyleneimine-polyethyleneglycol-N-acety- lgalactosamine (PEI-PEG-GAL) or polyethyleneimine-polyethyleneglycol-tri-N- -acetylgalactosamine (PEI-PEG-triGAL) derivatives. In one embodiment, the nucleic acid molecules of the invention

25 are formulated as described in U.S. Patent Application Publication No. 20030077829, incorporated by reference herein in its entirety.

In one embodiment, an oligonucleotide agent of the invention is complexed with membrane disruptive agents such as those described in U.S. Patent Application Publication No. 20010007666, incorporated by reference herein in its entirety

30 including the drawings. In another embodiment, the membrane disruptive agent or agents and the oligonucleotide agent are also complexed with a cationic lipid or

helper lipid molecule, such as those lipids described in U.S. Pat. No. 6,235,310,
incorporated by reference herein in its entirety including the drawings.

In one embodiment, an oligonucleotide agent of the invention is complexed
with delivery systems as described in U.S. Patent Application Publication No.
5   2003077829 and International PCT Publication Nos. WO 00/03683 and WO
02/087541, all incorporated by reference herein in their entirety including the
drawings.

In one embodiment, the nucleic acid molecules of the invention are
administered via pulmonary delivery, such as by inhalation of an aerosol or spray
10   dried formulation administered by an inhalation device or nebulizer, providing rapid
local uptake of the nucleic acid molecules into relevant pulmonary tissues. Solid
particulate compositions containing respirable dry particles of micronized nucleic acid
compositions can be prepared by grinding dried or lyophilized nucleic acid
compositions, and then passing the micronized composition through, for example, a
15   400 mesh screen to break up or separate out large agglomerates. A solid particulate
composition comprising the nucleic acid compositions of the invention can optionally
contain a dispersant which serves to facilitate the formation of an aerosol as well as
other therapeutic compounds. A suitable dispersant is lactose, which can be blended
with the nucleic acid compound in any suitable ratio, such as a 1 to 1 ratio by weight.

20   Aerosols of liquid particles comprising a nucleic acid composition of the
invention can be produced by any suitable means, such as with a nebulizer (see for
example U.S. Pat. No. 4,501,729). Nebulizers are commercially available devices
which transform solutions or suspensions of an active ingredient into a therapeutic
aerosol mist either by means of acceleration of a compressed gas, typically air or
25   oxygen, through a narrow venturi orifice or by means of ultrasonic agitation. Suitable
formulations for use in nebulizers comprise the active ingredient in a liquid carrier in
an amount of up to 40% w/w preferably less than 20% w/w of the formulation. The
carrier is typically water or a dilute aqueous alcoholic solution, preferably made
isotonic with body fluids by the addition of, for example, sodium chloride or other
30   suitable salts. Optional additives include preservatives if the formulation is not
prepared sterile, for example, methyl hydroxybenzoate, anti-oxidants, flavorings,

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volatile oils, buffering agents and emulsifiers and other formulation surfactants. The
aerosols of solid particles comprising the active composition and surfactant can
likewise be produced with any solid particulate aerosol generator. Aerosol generators
for administering solid particulate therapeutics to a subject produce particles which
are respirable, as explained above, and generate a volume of aerosol containing a
predetermined metered dose of a therapeutic composition at a rate suitable for human
administration. One illustrative type of solid particulate aerosol generator is an
insufflator. Suitable formulations for administration by insufflation include finely
comminuted powders which can be delivered by means of an insufflator. In the
insufflator, the powder, e.g., a metered dose thereof effective to carry out the
treatments described herein, is contained in capsules or cartridges, typically made of
gelatin or plastic, which are either pierced or opened in situ and the powder delivered
by air drawn through the device upon inhalation or by means of a manually-operated
pump. The powder employed in the insufflator consists either solely of the active
ingredient or of a powder blend comprising the active ingredient, a suitable powder
diluent, such as lactose, and an optional surfactant. The active ingredient typically
comprises from 0.1 to 100 w/w of the formulation. A second type of illustrative
aerosol generator comprises a metered dose inhaler. Metered dose inhalers are
pressurized aerosol dispensers, typically containing a suspension or solution
formulation of the active ingredient in a liquified propellant. During use these devices
discharge the formulation through a valve adapted to deliver a metered volume to
produce a fine particle spray containing the active ingredient. Suitable propellants
include certain chlorofluorocarbon compounds, for example,
dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethan- e and
mixtures thereof. The formulation can additionally contain one or more co-solvents,
for example, ethanol, emulsifiers and other formulation surfactants, such as oleic acid
or sorbitan trioleate, anti-oxidants and suitable flavoring agents. Other methods for
pulmonary delivery are described in, for example U.S. Patent Application No.
20040037780, and U.S. Pat. Nos. 6,592,904; 6,582,728; 6,565,885.

In one embodiment, nucleic acid molecules of the invention are administered
to the central nervous system (CNS) or peripheral nervous system (PNS).
Experiments have demonstrated the efficient in vivo uptake of nucleic acids by

neurons. As an example of local administration of nucleic acids to nerve cells,
Sommer et al., 1998, Antisense Nuc. Acid Drug Dev., 8, 75, describe a study in which
a 15mer phosphorothioate antisense nucleic acid molecule to c-fos is administered to
rats via microinjection into the brain. Antisense molecules labeled with

5   tetramethylrhodamine-isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC)
were taken up by exclusively by neurons thirty minutes post-injection. A diffuse
cytoplasmic staining and nuclear staining was observed in these cells. As an example
of systemic administration of nucleic acid to nerve cells, Epa et al., 2000, Antisense
Nuc. Acid Drug Dev., 10, 469, describe an in vivo mouse study in which beta-

10   cyclodextrin-adamantane-oligonucleotide conjugates were used to target the p75
neurotrophin receptor in neuronally differentiated PC12 cells. Following a two week
course of IP administration, pronounced uptake of p75 neurotrophin receptor
antisense was observed in dorsal root ganglion (DRG) cells. In addition, a marked and
consistent down-regulation of p75 was observed in DRG neurons. Additional

15   approaches to the targeting of nucleic acid to neurons are described in Broaddus et al.,
1998, J. Neurosurg., 88(4), 734; Karle et al., 1997, Eur. J. Pharmocol., 340(2/3), 153;
Bannai et al., 1998, Brain Research, 784(1,2), 304; Rajakumar et al., 1997, Synapse,
26(3), 199; Wu-pong et al., 1999, BioPharm, 12(1), 32; Bannai et al., 1998, Brain
Res. Protoc., 3(1), 83; Simantov et al., 1996, Neuroscience, 74(1), 39. Nucleic acid

20   molecules of the invention are therefore amenable to delivery to and uptake by cells in
the CNS and/or PNS.

The delivery of nucleic acid molecules of the invention to the CNS is provided
by a variety of different strategies. Traditional approaches to CNS delivery that can be
used include, but are not limited to, intrathecal and intracerebroventricular

25   administration, implantation of catheters and pumps, direct injection or perfusion at
the site of injury or lesion, injection into the brain arterial system, or by chemical or
osmotic opening of the blood-brain barrier. Other approaches can include the use of
various transport and carrier systems, for example though the use of conjugates and
biodegradable polymers. Furthermore, gene therapy approaches, for example as

30   described in Kaplitt et al., U.S. Pat. No. 6,180,613 and Davidson, WO 04/013280, can
be used to express nucleic acid molecules in the CNS.

In one embodiment, delivery systems of the invention include, for example, aqueous and nonaqueous gels, creams, multiple emulsions, microemulsions, liposomes, ointments, aqueous and nonaqueous solutions, lotions, aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers,

5   permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrolidone). In one embodiment, the pharmaceutically acceptable carrier is a liposome or a transdermal enhancer. Examples of liposomes which can be used in this invention include the following: (1) CellFectin, 1:1.5 (M/M) liposome formulation of the

10   cationic lipid N,NI,NII,NIII-tetramethyl-N,NI,NII,NIII- -tetrapalmit-y-spermine and dioleoyl phosphatidylethanolamine (DOPE) (GIBCO BRL); (2) Cytofectin GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (3) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-tri-methyl-ammoniummethylsulfate) (Boehringer Manheim); and (4) Lipofectamine, 3:1 (M/M) liposome formulation of

15   the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

In one embodiment, delivery systems of the invention include patches, tablets, suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and

20   hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

In one embodiment, oligonucleotide agents of the invention are formulated or complexed with polyethylenimine (e.g., linear or branched PEI) and/or polyethylenimine derivatives, including for example grafted PEIs such as galactose PEI, cholesterol PEI, antibody derivatized PEI, and polyethylene glycol PEI (PEG-

25   PEI) derivatives thereof (see for example Ogris et al., 2001, AAPA PharmSci, 3, 1-11; Furgeson et al., 2003, Bioconjugate Chem., 14, 840-847; Kunath et al., 2002, Phramaceutical Research, 19, 810-817; Choi et al., 2001, Bull. Korean Chem. Soc., 22, 46-52; Bettinger et al., 1999, Bioconjugate Chem., 10, 558-561; Peterson et al., 2002, Bioconjugate Chem., 13, 845-854; Erbacher et al., 1999, Journal of Gene

30   Medicine Preprint, 1, 1-18; Godbey et al., 1999., PNAS USA, 96, 5177-5181; Godbey et al., 1999, Journal of Controlled Release, 60, 149-160; Diebold et al., 1999, Journal of Biological Chemistry, 274, 19087-19094; Thomas and Klibanov, 2002, PNAS

USA, 99, 14640-14645; and Sagara, U.S. Pat. No. 6,586,524, incorporated by reference herein.

The present invention therefore includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, e.g., acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

The invention also features compositions comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic et al. Chem. Rev. 1995, 95, 2601-2627; Ishiwata et al., Chem. Pharm. Bull. 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic et al., Science 1995, 267, 1275-1276; Oku et al., 1995, Biochim. Biophys. Acta, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu et al., J. Biol. Chem. 1995, 42, 24864-24870; Choi et al., International PCT Publication No. WO 96/10391; Ansell et al., International PCT Publication No. WO 96/10390; Holland et al., International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

Methods of Modulating an Immune Response via IFN Production

The invention further provides a method for modulating an immune response in a vertebrate. The method comprises the step of administering to the vertebrate an immuno-stimulatory oligonucleotide of the invention.

As used herein, the term "vertebrate" includes, without limitation, a fish, bird, or mammal. As used herein, the term "mammal" includes, without limitation rats, mice, cats, dogs, horses, cattle, cows, pigs, rabbits, non-human primates, and humans.

As used herein, "modulating an immune response" means causing an increase

5   in, or activation of one or more of B-cell induction, T-cell induction, cytokine induction, natural killer cell induction, specific cell surface marker expression, chemokine induction and activation of antigen presenting cells, such as dendritic cells, monocytes and macrophages. Particularly, such immune response will involve the production of IFN in cells expressing the TLR7 protein, such as PDC.

10 The present invention further provides a method for treating a vertebrate having a disease that can be ameliorated by inducing IFN production. The method according to this embodiment of the invention comprises administering to the vertebrate an oligonucleotide agent of the present invention, e.g. an agent consisting of, consisting essential of or comprising SEQ ID NO:1. In such a use, an

15 oliognucloetide agent of the present invention is used to stimulate IFN production in a vertebrate to directly treat the condition or augment other therapies. There are well recognized clinical settings where selective activation of IFN production is wanted.

In another embodiment, the invention provides methods for therapeutically treating a patient having a disease or disorder, such methods comprising administering

20 to the patient an immunomodulatory oligonucleotide, immunomodulatory oligonucleotide conjugate, oligonucleotide agent or oligonucleotide agent conjugate according to the invention. In various embodiments, the disease or disorder to be treated is cancer, an autoimmune disorder, airway inflammation, inflammatory disorders, allergy, asthma or a disease caused by a pathogen. Pathogens include

25 bacteria, parasites, fungi, viruses, viroids and prions. Administration is carried out as described elsewhere herein.

As used herein, the term "allergy" includes, without limitation, food allergies and respiratory allergies. The term "airway inflammation" includes, without limitation, asthma.

As used herein, the term "autoimmune disorder" refers to disorders in which "self" proteins undergo attack by the immune system. Such term includes autoimmune asthma.

In any of the methods of the invention, the immunomodulatory oligonucleotide, immunomodulatory oligonucleotide conjugate, oligonucleotide agent or oligonucleotide agent conjugate can be administered in combination with any other agent useful for treating the disease or condition that does not diminish the immunostimulatory effect of the oligonucleotide agent. For example, in the treatment of cancer, it is contemplated that the immunomodulatory oligonucleotide, immunomodulatory oligonucleotide conjugate, oligonucleotide agent or oligonucleotide agent conjugate may be administered in combination with a chemotherapeutic compound

Examples of cellular proliferative and/or differentiative disorders include cancer, e.g., a carcinoma, sarcoma, metastatic disorder or hematopoietic neoplastic disorder, such as a leukemia. A metastatic tumor can arise from a multitude of primary tumor types, including but not limited to those of prostate, colon, lung, breast and liver origin. As used herein, the terms "cancer," "hyperproliferative," and "neoplastic" refer to cells having the capacity for autonomous growth, i.e., an abnormal state or condition characterized by rapidly proliferating cell growth. These terms are meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. Proliferative disorders also include hematopoietic neoplastic disorders, including diseases involving hyperplastic/neoplastic cells of hematopoietic origin, e.g., arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof.

The pharmaceutical compositions of the present invention can also be used to treat a variety of hematopoietic disorders. Examples of hematopoietic disorders or diseases include, without limitation, autoimmune diseases (including, for example, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, encephalomyelitis, myasthenia gravis, systemic lupus erythematosis, autommmune thyroiditis, dermatitis

34

(including atopic dermatitis and eczematous dermatitis), psoriasis, Sjogren's Syndrome, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema

5   nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing, loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves' disease, sarcoidosis, primary

10   biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis), graft-versus-host disease, cases of transplantation, and allergy.

In another embodiment, the invention relates to methods for treating viral diseases, including but not limited to hepatitis C, hepatitis B, herpes simplex virus (HSV), HIV-AIDS, poliovirus, and smallpox virus. Examples of (+) strand RNA

15   viruses include, without limitation, picornaviruses, caliciviruses, nodaviruses, coronaviruses, arteriviruses, flaviviruses, and togaviruses. Examples of picornaviruses include enterovirus (poliovirus 1), rhinovirus (human rhinovirus 1A), hepatovirus (hepatitis A virus), cardiovirus (encephalomyocarditis virus), aphthovirus (foot-and-mouth disease virus O), and parechovirus (human echovirus 22). Examples of

20   caliciviruses include vesiculovirus (swine vesicular exanthema virus), lagovirus (rabbit hemorrhagic disease virus), "Norwalk-like viruses" (Norwalk virus), "Sapporo-like viruses" (Sapporo virus), and "hepatitis E-like viruses" (hepatitis E virus). Betanodavirus (striped jack nervous necrosis virus) is the representative nodavirus. Coronaviruses include coronavirus (avian infections bronchitis virus) and

25   torovirus (Berne virus). Arterivirus (equine arteritis virus) is the representative arteriviridus. Togavirises include alphavirus (Sindbis virus) and rubivirus (Rubella virus). Finally, the flaviviruses include flavivirus (Yellow fever virus), pestivirus (bovine diarrhea virus), and hepacivirus (hepatitis C virus). In a preferred embodiment, the virus is hepacivirus, the hepatitis C virus.

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Pharmaceutical Compositions

In one embodiment, the invention relates to a pharmaceutical composition containing an oligonucleotide agent, as described in the preceding sections, and a pharmaceutically acceptable carrier, as described below. A pharmaceutical

5   composition including the oligonucleotide agent is useful for treating a disease that can be ameliorated by inducing IFN production. In this embodiment of the invention, the oligonucleotide agent of the invention is formulated as described below. The pharmaceutical compositions of the present invention are administered in dosages sufficient to induce IFN production. Where the pharmaceutical composition

10   comprises an iRNA agent, the dosage will also be sufficient to inhibit the expression or activity of the target gene. Compositions containing the oligonucleotide agent of the invention can be administered at surprisingly low dosages. A maximum dosage of 5 mg oligonucleotide agent per kilogram body weight per day may be sufficient to induce IFN production, and, where applicable, to inhibit or completely suppress the

15   expression or activity of the target gene.

Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents

20   can be provided. These include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

In general, a suitable dose of the oligonucleotide agent will be in the range of 0.001 to 500 milligrams per kilogram body weight of the recipient per day (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100

25   micrograms per kilogram to about 100 milligrams per kilogram, about 1 milligrams per kilogram to about 75 milligrams per kilogram, about 10 micrograms per kilogram to about 50 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram). The pharmaceutical composition may be administered once per day, or the oligonucleotide agent may be administered as two, three, four,

30   five, six or more sub-doses at appropriate intervals throughout the day. In that case, the oligonucleotide agent contained in each sub-dose must be correspondingly smaller

in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, e.g., using a conventional sustained release formulation which provides sustained release of the oligonucleotide agent over a several day period. Sustained release formulations are well known in the art. In this embodiment,

5   the dosage unit contains a corresponding multiple of the daily dose.

The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the infection or disease, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a

10   therapeutically effective amount of a composition can include a single treatment or a series of treatments. Estimates of effective dosages and in vivo half-lives for the individual oligonucleotide agent encompassed by the invention can be made using conventional methodologies or on the basis of in vivo testing using an appropriate animal model, as described elsewhere herein.

15   The pharmaceutical compositions encompassed by the invention may be administered by any means known in the art including, but not limited to oral or parenteral routes, including intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, airway (aerosol), ocular, rectal, vaginal and topical (including buccal and sublingual) administration. In preferred embodiments, the pharmaceutical

20   compositions are administered by intravenous or intraparenteral infusion or injection. The pharmaceutical compositions can also be administered intraparenchymally, intrathecally, and/or by stereotactic injection.

For oral administration, the oligonucleotide agent useful in the invention will generally be provided in the form of tablets or capsules, as a powder or granules, or as

25   an aqueous solution or suspension. Capsules for oral use include hard gelatin capsules in which the active ingredient is mixed with a solid diluent, and soft gelatin capsules wherein the active ingredient is mixed with water or an oil such as peanut oil, liquid paraffin or olive oil.

For intramuscular, intraperitoneal, subcutaneous and intravenous use, the

30   pharmaceutical compositions of the invention will generally be provided in sterile aqueous solutions or suspensions, buffered to an appropriate pH and isotonicity.

Suitable aqueous vehicles include Ringer's solution and isotonic sodium chloride. In a preferred embodiment, the carrier consists exclusively of an aqueous buffer. In this context, "exclusively" means no auxiliary agents or encapsulating substances are present which might affect or mediate uptake of oligonucleotide agent in the cells that

5   harbor the target gene or virus. Such substances include, for example, micellar structures, such as liposomes or capsids, as described below. Although microinjection, lipofection, viruses, viroids, capsids, capsoids, or other auxiliary agents are required to introduce oligonucleotide agents into cell cultures, surprisingly these methods and agents are not necessary for uptake of the oligonucleotide agent in

10   vivo. The oligonucleotide agents of the present invention are particularly advantageous in that they do not require the use of an auxiliary agent to mediate uptake of the oligonucleotide agent into the cell, many of which agents are toxic or associated with deleterious side effects. Aqueous suspensions according to the invention may include suspending agents such as cellulose derivatives, sodium

15   alginate, polyvinyl-pyrrolidone and gum tragacanth, and a wetting agent such as lecithin. Suitable preservatives for aqueous suspensions include ethyl and n-propyl p-hydroxybenzoate.

The nucleic acid molecules of the invention can also be administered in the form of suppositories, e.g., for rectal administration of the drug. These compositions

20   can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

The pharmaceutical compositions can also include encapsulated formulations

25   to protect the oligonucleotide agent against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in

30   the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as

pharmaceutically acceptable carriers. These can be prepared according to methods
known to those skilled in the art, for example, as described in U.S. Patent No.
4,522,811; PCT publication WO 91/06309; and European patent publication EP-A-
43075, which are incorporated by reference herein.

5   A preferred pharmaceutical composition according to the present invention is a
vaccine. A vaccine should contain an antigen besides the oligonucleotide according to
the present invention. The potential of this antigen to raise a protection/immune
response of the vaccinated individual is strongly increased by combining it with the
oligonucleotides according to the present invention, especially due to their
10   immunostimulatory activity.

        A vaccine can contain a whole variety of different antigens. Examples of
antigens are whole-killed organisms such as inactivated viruses or bacteria, fungi,
protozoa or even cancer cells. Antigens may also consist of subfractions of these
organisms/tissues, of proteins, or, in their most simple form, of peptides. Antigens can
15   also be recognised by the immune system in form of glycosylated proteins or peptides
and may also be or contain polysaccharides or lipids. Short peptides can be used since
for example cytotoxic T cells (CTL) recognize antigens in form of short usually 8-11
amino acids long peptides in conjunction with major histocompatibility complex
(MHC) (Rammensee et al., *Immunogenetics*, **41**, 178-228 (1995)). B cells recognize
20   longer peptides starting at around 15 amino acids (Harrow et al, Cold Spring Harbor:
Cold Spring Harbor Laboratory, (1988)). By contrast to T cell epitopes, the three
dimensional structure of B cell antigens may also be important for recognition by
antibodies. In order to obtain sustained, antigen-specific immune responses, adjuvants
are helpful to trigger immune cascades that involve all cells of the immune system
25   necessary. Primarily, adjuvants are acting, but are not restricted in their mode of
action, on so-called antigen presenting cells (APCs). These cells usually first
encounter the antigen(s) followed by presentation of processed or unmodified antigen
to immune effector. Intermediate cell types may also be involved. Only effector cells
with the appropriate specificity are activated in a productive immune response. The
30   adjuvant may also locally retain antigens and co-injected other factors. In addition the
adjuvant may act as a chemoattractant for other immune cells or may act locally
and/or systemically as a stimulating agent for the immune system.

The antigens to be used in the present compositions are not critical. Preferably, proteins or peptides derived from a viral or a bacterial pathogen or from fungi or parasites are used as such antigens (including derivatized antigens or glycosylated or lipidated antigens or polysaccharides or lipids). Another preferred source of antigens

5   are tumor antigens. Preferred pathogens are selected from human immunodeficiency virus (HIV), hepatitis A and B viruses, hepatitis C virus (HCV), rous sarcoma virus (RSV), Epstein Barr virus (EBV) Influenza virus, Rotavirus, Staphylococcus aureus, Chlamydia pneumonias, Chlamydia trachomatis, Mycobacterium tuberculosis, Streptococcus pneumonias, Bacillus anthracis, Vibrio cholerae, Plasmodium sp. (Pl.

10 falciparum, Pl. vivax, etc.), Aspergillus sp. or Candida albicans. Antigens may also be molecules expressed by cancer cells (tumor antigens). The derivation process may include the purification of a specific protein from the pathogen/cancer cells, the inactivation of the pathogen as well as the proteolytic or chemical derivatization or stabilisation of such a protein. In the same way also tumor antigens (cancer vaccines)

15 or autoimmune antigens may be used in the pharmaceutical composition according to the present invention. With such compositions a tumor vaccination or a treatment for autoimmume diseases may be performed.

In the case of peptide antigens the use of peptide mimitopes/ agonists/ superagonists/ antagonists or peptides changed in certain positions without affecting

20 the immunologic properties or non-peptide mimitopes/agonists/superagonists/antagonists (reviewed in Sparbier and Walden, *Curr Opin Immunol*, 11, 214-218 (1999)) is included in the current invention. Peptide antigens may also contain elongations either at the carboxy or at the amino terminus of the peptide antigen facilitating interaction with the polycationic compound(s) or the

25 immunostimulatory compound(s). For the treatment of autoimmune diseases peptide antagonists may be applied. Antigens may also be derivatized to include molecules enhancing antigen presentation and targeting of antigens to antigen presenting cells.

Toxicity and therapeutic efficacy of an oligonucleotide agent can be determined by standard pharmaceutical procedures in cell cultures or experimental

30 animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed

as the ratio LD50/ED50. oligonucleotide agents that exhibit high therapeutic indices are preferred.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosages of compositions of the invention are preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any oligonucleotide agent used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range of the oligonucleotide agent or of IFN, or, when appropriate, of the polypeptide product of a target sequence of an iRNA agent (e.g., achieving a decreased concentration of the polypeptide) that includes the IC50 (i.e., the concentration of the test oligonucleotide agent which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

In addition to their administration individually or as a plurality, as discussed above, oligonucleotide agents relating to the invention can be administered in combination with other known agents effective in treating viral infections and diseases. In any event, the administering physician can adjust the amount and timing of oligonucleotide agent administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Other embodiments are in the claims.

EXAMPLES

Example 1

Isolation of Plasmacytoid Dendritic Cells

PBMC were obtained from whole blood of healthy individuals by Ficoll-Hypaque density gradient centrifugation (Biochrom, Berlin, Germany). PDC were isolated by MACS using the BDCA-4 dendritic cell isolation kit from Miltenyi Biotec. Briefly, PDC were labeled with anti-BDCA-4 Ab coupled to colloidal paramagnetic microbeads and passed through a magnetic separation column once (LS column; Miltenyi Biotec). The purity of isolated PDC (lineage-negative, MHC-II-positive, and CD123-positive cells) was between 75 % and 100 %. Contaminating cells were mainly T cells. Viability was > 95 % as determined by trypan blue exclusion.

Cell Culture

Isolated PDC were cultured in 96-well flat-bottom plates at a concentration of 5×10^4 cells in 150 µl OPTIMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10 ng/ml IL-3 (R&D Systems, Wiesbaden, Germany). HEK 293 cells stably transfected with a construct containing the human TLR9 gene with a C-terminal yellow fluorescent protein (YFP) tag were kindly provided by D. Golenbock (Worcester, MA). HEK 293 cells were cultured in RPMI 1640 culture medium (Biochrom) supplemented with 10% (v/v) fetal calf serum (BioWhittaker, Walkersville, MD), 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Sigma-Aldrich, Munich, Germany). EBV-negative Burkitt's lymphoma cell line BL41 was grown in RPMI 1640 (PAA, Linz, Austria), supplemented with 10 % (v/v) fetal calf serum (Biochrom), 2 mM L-glutamine, 0.1 mg/ml penicillin/streptomycin and 1 % 1 mM pyruvate (all from PAA). Cells were incubated at 37°C and 5 % CO_2.

In Vitro Cell Stimulation, Transfection and Electroporation

CpG ODN were provided by Coley Pharmaceutical Group (Wellesley, MA) (underlined letters, phosphorothioate linkage 3' of the base; bold letters, CpG dinucleotides):

5　　　　　ODN 2216: 5'-GGGGGACGATCGTCGGGGGG-3';　　　　　SEQ ID NO:7

　　　　　　ODN 1826: 5'-TCCATGACGTTCCTGACGTT-3'.　　　　　　SEQ ID NO:8

R848 was purchased from Invivogen (Toulouse, France). R848 and ODN 2216 were added at a final concentration of 500 ng/ml and 3 µg/ml respectively. The siRNA sequences (see table 1) were synthesized and annealed by Dharmacon

10　　　(Lafayette, CO). siRNA was transfected with lipofectamine 2000 (Invitrogen, Karlsruhe, Germany). If not indicated otherwise, 200 ng of nucleic acid were mixed with 25 µl of OPTIMEM. In a separate tube, 0.5 µl of lipofectamine was added to 25 µl of OPTIMEM and incubated for 5 min at room temperature. For complex formation, both solutions were mixed, incubated for an additional 20 min at room

15　　　temperature, and added to cells (100 µl) in a 96-well plate (final volume 150 µl). Cells in transfection solution were incubated at 37°C without additional washing.

For electroporation, 250.000 PDC were resuspended in 400µl "isoosmolar electroporation-buffer" (Eppendorf, Hamburg, Germany) with or without siRNA (2,5 µg/ ml) and pulsed once with at 100V for 50µs (Multiporator, Eppendorf). After 5

20　　　minutes 800µl complete medium was added and cells were incubated in 24-well flat bottom wells for additional 36h.

Mouse Studies

For *in vivo* stimulation, 129P2/OlaHsd or 129Sv mice were anesthetized and 200 µl containing 5 µg CpG ODN 1826 or siRNA TLR9.2 with or without prior 1,2-

25　　　dioleoyloxy-3-trimethylammonium-propane (DOTAP)-complexation (30 µl DOTAP (Boehringer Mannheim) were mixed with 5 µg of nucleic acids in 170 µl of Hepes-buffered saline (HBS)) were injected i.v. into the retro-orbital vein. Control mice received either HBS alone (control) or HBS and DOTAP (DOTAP). Whole blood samples were obtained by tail clippings at either 7 hours, 18 hours or 24 hours after

injection. Serum was prepared from whole blood by coagulation for 30 min at 37°C
and subsequent centrifugation.

<u>Detection of Cytokines</u>

5   Because total IFN-α is comprised of 14 different isoforms, the quantity of
IFN-α measured by ELISA depends on the specificity of the antibody used for the
detection of these isoforms, and thus is not identical between different ELISA. For
this study, the IFN-α kit by Bender MedSystems (Graz, Austria) (detection range 8–
500 pg/ml) was used. This ELISA detects most of IFN-α isoforms, but not IFN-B and
IFN-F. The human TNF-α ELISA (detection range 8 to 500 pg/ml) and the human IL-
10   6 ELISA (detection range 5 to 300 pg/ml) were from BD PharMingen (Heidelberg,
Germany). Murine IFN-α was determined using the mouse IFN-α ELISA kit from
PBL Biomedical Laboratories (Piscataway, USA). All ELISA procedures were
performed as recommended by the manufacturer.

<u>Flow Cytometry</u>

15   Flow cytometric data were acquired on a BD Biosciences FACSCalibur
(Heidelberg, Germany) equipped with two lasers (excitation at 488 nm and 635 nm
wavelength). In general, cells were stained for 20 min at 4°C with the indicated
specific antibodies and the appropriate isotype controls. Human PDC were identified
by positive staining with anti-CD123 PE and anti-MHC II PerCP and negative
20   staining with anti-lineage FITC. Costimulatory molecule expression was determined
by using anti-CD86 APC (all BD PharMingen). For PDC survival, cells were
harvested 36 h after stimulation and the absolute number of viable cells per 50 μl
aspirated volume was determined by flow cytometry. Viability of PDC was
determined by staining negative for TO-PRO-3 iodide (Molecular Probes, Eugene,
25   OR) and a morphology-based live gate. For phenotypic analysis of murine MDC
($CD11c^{++}$, $CD11b^{++}$ and $B220^-$) and PDC ($CD11c^+$, $CD11b^-$ and $B220^{++}$) freshly
isolated spleen cells were stained with anti-CD86 FITC, anti-CD45R/B220 PE, anti-
CD11b PerCP and anti-CD11c APC (all BD PharMingen). Activation of splenic NK
cells and T cells was determined by anti-CD69 PE on anti-pan-NK FITC-positive
30   cells (NK cells) and anti-CD3 APC-positive cells (T cells) (all BD PharMingen). Data

were analyzed using CellQuest (BD Biosciences) or FlowJo software (version 2.5.1;
Tree Star, Stanford, CA).

Western-Blot Analysis

The cells were washed once in phosphate-buffered saline (PBS) counted and
lysed by resuspension in Laemmli sample buffer (10^6 cells/50μl) followed by 10 sec
of sonication. The samples were separated on SDS-polyacrylamide gels (10 %) and
transferred onto nitro-cellulose membranes (Hybond-ECL, Amersham). Following
transfer, the membranes were stained in Ponceau Red to verify that equal amounts of
protein were loaded. The membranes were then washed in TBST (tris 10 mM pH 8.0,
NaCl 30 mM, 0.1% Tween) for 5 min and incubated for 30 min in TBST-MLK
(TBST supplemented with 5% dried skimmed milk). After rinsing with water twice,
specific antibodies (1:1000) in TBST-MLK were added for 1 h or overnight at 4°C.
After rinsing twice with water and three washes of 5 min with TBST, the secondary
antibodies conjugated with horseradish peroxidase (1 : 3000 in TBS-MLK) were
added for 1.5 h, followed by 3 times rinsing with water, three washes of 5 min in
TBST and 5 times rinsing with water. Bands were visualized on ECL films
(Amersham) by enhanced chemiluminescence following the supplier's procedure
(Amersham). Antibodies to the following proteins were used: phospho (tyrosine 701)-
STAT1 (New-England Biolabs, Beverly, MA) and phospho (tyrosine 689)-STAT2
(Upstate, Waltham, MA).

Statistical Analysis

Data are expressed as means ± SEM. Statistical significance of differences
was determined by the paired two-tailed Student's t-test. Differences were considered
statistically significant for $p < 0.05$. Statistical analyses were performed using
StatView 4.51 software (Abacus Concepts Inc., Calabasas, CA).

Specific siRNA-Mediated Inhibition of TLR9 Expression in HEK 293 Cells

The plasmacytoid dendritic cell (PDC) has been identified as the key sensor of
CpG motifs. Selective inhibition of TLR9 in PDC with siRNA may allow analysis of
the involvement of TLR9 in the recognition of viruses and of different types of CpG

45

motif containing oligodesoxynucleotides (CpG ODN). We established siRNA-mediated downregulation of TLR9 in a HEK 293 cell line stably transfected with a construct containing the human TLR9 gene with a C-terminal yellow fluorescent protein (YFP) tag. Four siRNA molecules (TLR9.1, TLR9.2, TLR9.3, TLR9.4)

5   targeting the human TLR9 mRNA were designed (Table 1). A standard BLAST-search ensured that these siRNA target sequences had no homologies with other human genes. siRNA targeting the human TLR2 mRNA was used as negative control, and a previously established siRNA targeting the C-terminal YFP tag of the TLR9 construct was used as a positive inhibition control (see Table 1). Adherent HEK

10   293cells were transfected with 200 ng siRNA using lipofectamine in 96-well plates. After 20 hours, 30 hours and 54 hours, HEK 293cells were harvested and expression of TLR9 was assessed by quantifying YFP fluorescence intensity by flow cytometry. At all three times points analyzed, two of the four TLR9-specific siRNA tested (TLR9.2 and TLR9.3) showed a strong inhibition of the TLR9-YFP fusion protein

15   expression that was in the same range as the anti-YFP siRNA used as a positive inhibition control (TLR9 expression after 20 hours shown in Fig. 1A; 30 hours: TLR2.1: 151 ± 10; TLR9.1: 71 ± 10; TLR9.2: 43 ± 2; TLR9.3: 48 ± 2; TLR9.4: 119 ± 5; GFP: 37 ± 3; 54 hours: TLR2.1: 167 ± 8, TLR9.1: 65 ± 4; TLR9.2: 32 ± 1; TLR9.3: 34 ± 2; TLR9.4: 110 ± 7; GFP: 24 ± 1).

20   In order to exclude the possibility that downregulation of the target gene in HEK 293 cells is associated with non-target specific type I IFN induction, we analyzed the supernatants of HEK 293 cells for type I IFN activity. The induction of STAT1 and STAT2 phosphorylation in BL41 cells was used as a highly sensitive measure of type I IFN. No type I IFN activity was detected in the supernatants of

25   HEK 293 cells transfected with the four different anti-TLR9 siRNA TLR9.1, TLR9.2, TLR9.3, TLR9.4 (Fig. 1B). As expected, 500 bp long dsRNA complexed with lipofectamine (but not without lipofectamine) induced similar STAT1 and STAT2 phosphorylating activity as obtained with the positive control recombinant IFN-β (Fig. 1B). These results demonstrated that two anti-TLR9 siRNA, TLR9.2 and

30   TLR9.3, allow specific downregulation of TLR9 expression in HEK 293 cells, and that in this cell line, transfection with siRNA does not induce type I IFN as a non-target-specific effect.

Sequence Dependent Potent Induction of IFN-α by siRNA in Plasmacytoid
Dendritic Cells

     Next we studied whether anti-TLR9 siRNA that inhibited TLR9 expression in
HEK 293 cells desensitizes PDC for stimulation with a TLR9 ligand. Since the PDC
5   is a major producer of type I IFN in the human immune system, the use of double-
stranded (ds) RNA for selective target protein inhibition in PDC might be complicated
by dsRNA-mediated non-target-specific type I IFN induction. To study this
possibility, PDC from human peripheral blood were incubated with either poly(I:C),
the prototype stimulus for dsRNA, or with a 500 basepairs (bp) long dsRNA
10   molecule; both were complexed with lipofectamine for cytosolic delivery. The TLR9
ligand CpG-A ODN 2216 (Krug, A. et al. *Eur J Immunol* 31, 2154-63 (2001)) and the
TLR7 ligand R848 served as positive controls. We found that both dsRNA molecules
lacked the ability to induce IFN-α in PDC, while the positive controls CpG-A ODN
2216 and R848 showed a vigorous IFN-α response. These results suggested that
15   dsRNA may be useful for selective knockdown of target genes in PDC.

    Surprisingly, transfection of the four anti-TLR9 siRNAs at 200 ng induced a
consistent pattern of IFN-α production in PDC (Fig. 2A). TLR9.2 and TLR9.3
induced significantly more IFN-α production in PDC than TLR9.1 and TLR9.4 (Fig.
2A). To exclude that RNA degradation is responsible for the lower degree of
20   interferon-induction by TLR9.1 and TLR9.4 (see Fig. 2A), we compared decreasing
amounts of all four sequences. At 25 ng (12.5 nM) the two sequences TLR9.2 and
TLR9.3 still induced higher levels of IFN-α than TLR9.1 at 200 ng (100 nM) (Fig.
2B), confirming the distinct activity of the four anti-TLR9 siRNA in inducing IFN-α
in PDC. Distinct IFN-α inducing activity of TLR9.1 and TLR9.2 was also observed
25   when siRNA was delivered into the cytosol of PDC by electroporation demonstrating
that complex formation of siRNA with lipofectamine is not required for the IFN-α
induction by siRNA; rather, the siRNA molecule itself seemed to be the active agent.
No IFN-α induction by siRNA was observed in B cells or myeloid dendritic cells
(data not shown).

30   Of note, among the four anti-TLR9 siRNAs tested, the two with the highest
activity to downregulate TLR9 expression in HEK 293 cells were also the most potent

sequences to induce IFN-α in PDC (compare Fig. 1A and Fig. 2A). Although
unlikely, a target-specific effect (inhibition of TLR9) rather than non-target-specific
immunostimulatory effect may be responsible for the IFN-α induction in PDC. To
exclude this possibility we compared a panel of siRNAs targeting mRNA sequences

5   that are expressed in PDC (TLR9) or not expressed in PDC (TLR2, TLR3, TLR4, see
Table 1). Considerable differences in the potency of these siRNAs to induce IFN-α
were seen, but no correlation was found between the potency to induce IFN-α and the
presence or absence of the target mRNA in PDC (ODN 2216: x ADD VALUE±SEM;
from low to high IFN-α induction: TLR4.1: 491±59; TLR9.1: 500±88; TLR9.4:

10   760±131; TLR4.2: 1208±267; TLR3.2: 1219±204; TLR2.1: 1535±235; TLR3.1:
2503±552; TLR9.3: 3592±283; TLR9.2: 4845±621: n=6). The possibility of
differences in preparation quality or contaminants contributing to distinct IFN-α -
inducing activity of siRNA was excluded by using siRNA sequences from different
commercial sources that gave identical results (data not shown). These data indicated

15   that IFN-α induction in PDC by siRNA is sequence-dependent and is not linked to the
presence or absence of the target mRNA.

Identification of an IFN-α -Inducing RNA Sequence

It has been reported that guanosine- and uridine rich single-stranded RNA
induces IFN-α in PDC. A mixture of guanosine and uridine monomers also showed

20   immunostimulatory activity. Therefore we speculated that differences in the
guanosine and uridine content of the siRNA in our study may be responsible for the
different activity of siRNA to induce IFN-α in PDC. Since siRNA is double-stranded,
the total content of guanosine and uridine in a 19mer siRNA is always 19. The
number of uridines in the siRNAs tested ranged from 9 to 11 with no correlation

25   between number of uridines and IFN-α inducing activity (see Table 1). Immunoactive
siRNAs may still contain specific single strands (sense or anti-sense) with different
guanosine- and uridine content. However, the total numbers of guanosines and
uridines ranged between 7 and 12 in the single strands of the active siRNAs (TLR9.2
and TLR9.3) as well as in single strands of siRNAs with lower activity (TLR9.1,

30   TLR9.4, TLR2.1, TLR4.1, TLR4.2, TLR3.1, TLR3.2); furthermore, the ratio of
uridines to guanosines was not linked to the immunological activity (see Table 1).
Therefore, a specific sequence rather than simply the content of guanosines and

uridines seemed to be responsible for the immunological activity of TLR9.2 and
TLR9.3.

In order to identify the immunostimulatory sequence, we compared the IFN-α
inducing activity of the two single strands (sense and antisense) of TLR9.2 with the
5   activity of the duplex. The sense strand of TLR9.2 was equally potent to induce IFN-α
in PDC than the duplex, while the antisense strand of TLR9.2 showed only weak
activity (Fig. 2C, left panel). IFN-α induction by poly(U) or by poly(A) ssRNA and
by poly(U:A) dsRNA was low as compared to the sense strand of TLR9.2 siRNA
(RNA9.2s: 4962±797; poly(U): 574±165; poly(A): 244±120; poly(U:A): 111±53;
10   n=7; for poly(A) also see Fig. 3B second bar from left) further supporting the concept
that the sense strand of TLR9.2 contains a specific immunostimulatory motif that is
recognized by PDC.

Since siRNA is generated by annealing of the sense and the antisense strand,
the immunostimulatory activity of TLR9.2 siRNA may be due to unbound sense
15   strand in the siRNA preparation and not due to the duplex. To exclude this possibility,
the sense strand and the duplex of TLR9.2 were exposed to single-strand RNAse. As
expected, the sense strand was completely degraded by RNAse treatment (Fig. 2D,
left panel), leading to the loss of immunological activity (Fig. 2D, right panel). In
contrast, the duplex was not affected by RNAse treatment, and the activity to induce
20   IFN-α was maintained (Fig. 2D). To further exclude a contribution of contaminating
single-stranded RNA in the siRNA preparation we designed a single-stranded RNA
that forms an energetically stable hairpin (similar to microRNA) in which the double-
stranded region contains the complete duplex of TLR9.2 siRNA (Table 2). This
hairpin was found to be as active in inducing IFN-α as the TLR9.2 siRNA duplex
25   (Fig. 2E). These data demonstrated that immunostimulatory activity of siRNA cannot
be prevented by eliminating single-stranded contaminants in siRNA preparations, and
that immunostimulation is also relevant for the hairpin-based RNA interference (ie.
microRNA).

The sequences of TLR9.2 and TLR9.3 show an overlap of 13 bases (see Table
30   1). Since both siRNAs were potent inducers of IFN-α in PDC, we hypothesized that
the sequence motif responsible for IFNα induction is located in the overlapping region

of TLR9.2 and TLR9.3. Thus localization of the immunostimulatory motif pointed to
the 13 bases at the 3′end of the sense strand of TLR9.2. This assumption was
supported by decreased activity of the sense strand when a FITC molecule was linked
to the 3′end but not the 5′end (Fig. 3A, compare 5′F to 3′F). The lack of

5   immunological activity of poly(A) (Fig. 3A) allowed us to substitute bases in the
sense strand of TLR9.2 with A in order to narrow the motif. Consistent with a
localization of the immunostimulatory motif within the 13 bases of the 3′end of the
sense strand of TLR9.2, substitution of 8 bases at the 3′end (R8A, R stands for right)
but not at the 5′end (L8A, L stands for left) abolished the immunological activity of

10   the sense strand (Fig. 3B). By further increasing the number of A from the 5′end
(L8A to L12A), position 11 was identified to be essential for the motif at the 3′end,
suggesting that the 9 bases at the 3′end (5′GUCCUUCAA 3′, SEQ ID NO:1) of the
sense strand of TLR9.2 are responsible for its immunostimulatory activity. The
exchange of increasing numbers of bases in this 9mer sequence resulted in a gradually

15   decreasing immunological activity of the sense strand of TLR9.2 with a complete loss
of activity when 6 bases were exchanged within the 9mer sequence (Fig. 3B; R8A).
The minimal length of the RNA required for IFNα induction was found to be in the
range of 19 bases, since both a 12mer and a 16mer containing the 9mer sequence
showed a much lower activity (Fig. 3B). On the other hand, by placing the 9mer

20   sequence twice in a 19mer RNA oligonucleotide (termed DR), the immunological
activity was further increased as compared to the sense strand of TLR9.2, which only
contains one immunostimulatory 9mer sequence (termed n; Fig. 3B).

Dissection of Immunostimulatory Activity and Silencing Activity of siRNA

According to the above studies, the immunostimulatory activity of TLR9.2 is
25   localized in the sense strand and the silencing activity in the anti-sense strand of the
duplex. Appropriate modifications of both single RNA strands of TLR9.2 may allow
dissection of the immunostimulatory from the silencing activity and vice versa. The
backbone modification, locked nucleic acids (LNA), has been used to change the
properties of siRNA with regard to target inhibition. We were interested whether

30   LNA modification of the sense and the anti-sense strand can be used to modulate not
only the silencing but also the immunological activity of siRNA. First we examined
the impact of 5′and 3′ LNA on the immunological activity of the sense strand of

TLR9.2 (see Table 2). Consistent with linking a FITC molecule to the 5′ or the 3′ end
(see Fig. 3A), LNA modification at the 3′ end but not at the 5′ end strongly inhibited
the activity of the sense strand to induce IFNα (Fig. 4A). Next, sense and anti-sense
strand with different LNA modifications (LNA at 5′ end, at 3′ end, or at both 5′ and

5   3′ ends) or without LNA modification were annealed to generate a total of 16 different
TLR9.2 siRNA derivatives. We assessed the activity of this panel of TLR9.2 duplexes
to induce IFNα in PDC (Fig. 4B, black bars). Like for the sense strand alone (Fig.
4A), duplexes with LNA modification of the sense strand at the 3′ end or at both the
3′ end the 5′ end strongly inhibited IFNα inducing activity, while LNA modification at

10   the 5′ end sense strand showed no major changes of the activity (Fig. 4B). LNA
modifications of the anti-sense strand did not influence the immunostimulatory
activity of an siRNA containing an unmodified sense strand (Fig. 4B).

Next we examined the influence of LNA-modifications on the silencing
activity of TLR9.2 siRNA. Unlike for IFNα induction, LNA modification of the sense

15   strand had almost no effect on the silencing activity of TLR9.2 siRNA (Fig. 4B, open
bars). In contrast, LNA modification of the anti-sense strand strongly affected the
silencing activity of the TLR9.2 siRNA. LNA modification of the 3′ end of the anti-
sense strand had less impact on the silencing activity than LNA modification of the
5′ end of the anti-sense strand. Silencing activity was completely lost when both the 3′

20   and the 5′ ends were modified by LNA. Together these data indicated that it is
possible to dissociate the two functional activities of siRNA by introducing LNA
modifications to different regions of the sense and the anti-sense strand: the silencing
activity can be maintained while minimizing the immunostimulatory component of
siRNA (for example: LNA at the 3′ end of the sense strand); the immunostimulatory

25   activity can be maintained while abolishing the silencing activity of siRNA (i.e. LNA
at the 3′ and the 5′ end of the anti-sense strand).

Immunostimulatory siRNA Induces Systemic Immune Responses in Mice *In
Vivo*

In order to study the systemic activity of siRNA *in vivo*, we first examined the

30   *in vitro* immunostimulatory activity of siRNA sequences in the murine system.
Similar to the human system, the TLR9.2 duplex and the sense strand were found to

induce IFNα , while the anti-sense strand of TLR9.2 was much less active, and
poly(A) was inactive (Fig. 5A).

5   Next, the immunological activity of the TLR9.2 duplex, the TLR9.2 sense
strand, and the TLR9.2 anti-sense strand was assessed *in vivo*. TLR2.1 was included
in this analysis as a siRNA that in the human system was less active than TLR9.2.
Seven hours after i.v. injection into 129Sv mice, the concentration of IFN-α was
measured in the serum of mice (Fig. 5B). In addition, activation of immune cell
subsets in the spleen was assessed by flow cytometry after 43 hours (Fig. 5C).
Consistent with the *in vitro* data, the TLR9.2 duplex and the TLR9.2 sense strand
10   showed the highest activity to induce systemic levels of IFN-α and to activate CD4
and CD8 T cells and myeloid dendritic cells (Fig. 5C). No immunological activity
was observed with either cationic liposomes (DOTAP) or siRNA alone. (Fig. 5D).
Together, these results demonstrated that administration of siRNA *in vivo* elicits
systemic immune responses detectable in both blood and spleen and that the
15   immunological activity in mice shows similar sequence dependency as in the human
system.

Immune Recognition of siRNA Requires TLR7

    Conserved sequence dependent immune recognition of siRNA in mice and
humans allowed the use of knockout mice in order to identify the responsible
20   receptor. TLR7 and TLR9 are the only members of the TLR family that are expressed
in PDC FIX REFERENCE{Krug, 2001 #5;Hornung, 2002 #37} and that are known to
be linked to IFN-α production. So far, RNA sequence motifs specifically detected via
TLR7 have not been identified. We hypothesized that TLR7 is involved in the
recognition of the immunostimulatory sequence motif contained in TLR9.2 siRNA.
25   Indeed we found that IFN-α induction *in vitro* by the TLR9.2 duplex and the TLR9.2
sense strand as well as by an established TLR7 ligand (loxoribine) was absent in bone
marrow cells derived from TLR7-deficient mice (Fig. 6A). In contrast, IFN-α
induction by the TLR9 ligand CpG ODN 1826 was not reduced in bone marrow cells
derived from TLR7-deficient mice (Fig. 6A).

30   Additional *in vivo* mechanisms of immune recognition of siRNA might exist
that are not detectable *in vitro*. As such, TLR9.2 siRNA was injected i.v. into TLR7-

deficient mice and wild type control mice. In contrast to wild type mice, TLR7-
deficient mice had no detectable IFN-α in serum after 7 h and 24 h (Fig. 6B).
Furthermore, no activation of CD4 T cells, CD8 T cells, myeloid dendritic cells and
PDC was found in spleen cells of TLR7-deficient mice, while strong activation of all

5   four cell subsets was observed in wild type mice (Fig. 6C). Together these data
indicated that TLR7 is required for immune recognition of TLR9.2 siRNA.

Discussion

        One of the most significant advances in RNA interference technology was the
·observation that by reducing the length of the dsRNA molecules down to 22 bp, PKR-

10   mediated nonspecific type I IFN induction can be abolished while sequence-specific
downregulation of the target mRNA is maintained. In agreement with this concept we
were able to establish siRNA-mediated sequence-specific downregulation of TLR9 in
the human HEK293 cell line in the absence of an IFN response. However, when we
attempted to apply this method to primary plasmacytoid dendritic cells (PDC) we

15   made the surprising observation that the previously proposed length dependence of
dsRNA recognition seemed to be reversed: while transfection of PDC with a 500 bp
long dsRNA molecule or with poly(I:C) (mimicking long dsRNA) failed to induce
IFN-α, some of the siRNA sequences tested showed a vigorous IFN-α response.

        Based on the overlapping sequence of two siRNAs with potent IFN-α inducing

20   activity we were able to identify an immunostimulatory single-stranded RNA
sequence consisting of 9 bases (5'GUCCUUCAA 3', SEQ ID NO:1). By chemical
backbone modification of different regions of both strands of the immunostimulatory
siRNA, we were able to dissect immunostimulation and RNA silencing as two
independent functional activities. The same type of sequence-specificity with regard

25   to IFN-α induction by siRNA was seen in the mouse. *In vitro* immunostimulatory
activity and *in vivo* systemic immune responses were abrogated in TLR7-deficient
mice, demonstrating that sequence-specific immune recognition of siRNA is mediated
via TLR7.

        Our results provide evidence that the immunostimulatory activity of siRNA is

30   not mediated by single-stranded RNA molecules left over in the siRNA preparation
after an incomplete annealing process: i) treatment of the siRNA preparation with

single-strand RNAse did not affect the immunological activity of siRNA, while the
activity of the corresponding immunostimulatory single-strand RNA was abolished;
ii) a single-strand RNA oligonucleotide designed to spontaneously form an
energetically stable hairpin (in the form of microRNA) containing the complete
duplex of the immunostimulatory siRNA was as active as the immunostimulatory
siRNA. These data confirmed that recognition mechanisms responsible for the
recognition of siRNA allow the detection of a single-strand RNA motif within the
RNA duplex.

It is interesting to note that for the recognition of siRNA and the TLR9 ligand
(CpG DNA), the detection of the immunostimulatory sequence motifs occurs on the
single-strand level although both siRNA and microbial DNA are primarily double-
stranded (viral or bacterial DNA genomes are usually double-strand). To date there is
no information whether helicase activity is part of the recognition process. For both
RNA and DNA, synthetic single-stranded oligonucleotides containing the appropriate
sequence motifs can be used to elicit the corresponding type of immune responses.
The minimal length of an immunostimulatory RNA oligonucleotide was
approximately 19 bases. Similarly, the 6mer CpG motif needed to be part of a longer
oligonucleotide to become an active TLR9 ligand (Hartmann, G. et al. *J Immunol*
2000, 164:944-53). Since poly A RNA showed no immunological activity, the
addition of poly A could be used for elongation of an RNA oligonucleotide containing
the 9mer stimulatory sequence up to the length of a 19mer. The insertion of two
stimulatory 9mer sequences in a 19mer RNA oligonucleotide further increased the
immunostimulatory activity. By analogy, poly(C) was used in previous studies for
elongation of a CpG motif containing oligodesoxynucleotide, since poly(C) DNA
(with and without phosphorothioate modification) lacked immunostimulatory activity
(Hartmann, G. et al. *J Immunol* 2000, 164:944-53).

It has been reported that certain single-stranded RNA viruses such as vesicular
stomatitis virus and influenza virus are recognized by immune cells via TLR7 (Lund,
et al., Proc. Natl. Acad. Sci. USA 2004, 101:5598-603; Heil et al., Science 2004,
303:1526-9; Diebold, et al., Science 2004, 303:1526-9), but specific sequence motifs
responsible for viral RNA recognition have not been identified so far. Instead,
poly(U) has been reported to be active, and it has been proposed that GU-rich

sequences in viral RNA are responsible for immune recognition; furthermore, that a mixture of monomeric Gs and Us is immunostimulatory, indicating that RNA degradation products might be involved. There are several lines of evidence that the immunostimulatory activity of the RNA molecules in our study is not due to GU content. First, the total number of G and U of duplex RNA by definition is always identical with the length of the duplex (i.e. the GU count of a 19mer duplex is always 19). Second, a higher number of Gs or Us or the ratio of G to U in the one or the other of the two single strands of siRNA was not associated with the immunological activity of siRNA. Third, increasing the number of G in the immunostimulatory single strand RNA reduced rather than enhanced its immunological activity; and fourth, poly(U) was weak compared to the RNA oligonucleotide containing the immunostimulatory RNA sequence described in this study.

Although our results clearly demonstrate that immune recognition of siRNA by PDC is sequence-dependent and GU content-independent, the sequence described in this study may only be one of several different immunostimulatory RNA sequence motifs. Optimization of the sequence of this study and the identification of additional sequences will be the subject of a broader screen of sequences to identify the most potent immunostimulatory RNA motifs.

At least for the RNA sequence described in this study, recognition of siRNA seems to be conserved in mouse and man. Upon intravenous injection of immunostimulatory siRNA complexed with DOTAP we observed strong systemic immune responses including IFN-α in the serum and activation of T cells, PDC and myeloid dendritic cells. The level of immune activation was in the same range as with the TLR9 ligand CpG. Activation of myeloid dendritic cells and T cells by siRNA *in vivo* may be due to secondary effects mediated by IFN-α as has been demonstrated in the human system (Krug, A. et al. *J Immunol* 170, 3468-77 (2003), Rothenfusser, S. et al. *Blood* 103, 2162-9 (2004), Rothenfusser, S. et al. *Eur J Immunol* 31, 3525-34 (2001)). As a consequence cationic lipid-complexed siRNA, similar to CpG, may elicit potent therapeutic activity against viral (Dittmer, U. & Olbrich, A.R. *Curr Opin Microbiol* 6, 472-7 (2003)) and bacterial infection (Wagner, H., *Springer Semin Immunopathol* 22, 147-52 (2000)) and tumors (Heckelsmiller, K. et al. *Eur J Immunol* 32, 3235-45 (2002), Heckelsmiller, K. et al. *J Immunol* 169, 3892-9 (2002), Weiner,

G.J. *Curr Top Microbiol Immunol* 247, 157-70 (2000)). It is interesting to note that siRNA was successfully used to inhibit viral replication and transcription in a murine model of hepatitis B (Klein, C. et al. *Gastroenterology* 125, 9-18 (2003), Davidson, B.L. *N Engl J Med* 349, 2357-9 (2003)). Since IFN-α is a mainstay of hepatitis B

5   treatment, PDC-derived IFN-α might have contributed to therapeutic effects of siRNA.

In our study, immune responses induced by siRNA both *in vitro* and *in vivo* completely depended on the presence of TLR7. However, in many cell lines, such as HEK293 cells, TLR7 is not expressed and thus immune recognition of siRNA via

10   TLR7 is not expected. This is consistent with our *in vitro* observation that the supernatant of siRNA-transfected HEK293 cells, in contrast to transfection with long dsRNA molecules, shows no type I IFN response as evidenced by the lack of STAT1- or STAT2-phosphorylating activity. These results are in agreement with the concept, that in TLR7-deficient cell lines *in vitro*, short dsRNA of 21 bp (siRNA) lack

15   immunostimulatory activity and are useful for sequence-specific downregulation of target genes.

Contradicting this concept is a report suggesting that transfection of siRNAs into cell lines results in PKR-dependent type I IFN-mediated activation of the STAT pathway and leads to upregulation of IFN-stimulated genes (Sledz, C.A., et al. *Nat*

20   *Cell Biol* 5, 834-9 (2003)). Furthermore, siRNA was reported to induce type I IFN responses via TLR3 expressed in cell lines, including HEK293 cells (Kariko, K., et al. *J Immunol* 172, 6545-9 (2004), Kariko, K. et al. *Cells Tissues Organs* 177, 132-8 (2004)). However, the complete loss of immunostimulatory activity of siRNA in TLR7-deficient mice in our study provides evidence that PKR and TLR3 do not

25   represent the major mechanisms by which siRNA is detected by the innate immune system. This is supported by our recent finding that the immunological activity of siRNA is not affected in TRIF-deficient mice (data not shown). TRIF is a necessary adaptor molecule for TLR3 signaling. The lack of immunological activity of siRNA in TRIF-deficient mice also indicates that the alternative TLR3- and TRIF-

30   independent pathway of long double-stranded RNA (poly[I:C]) recognition proposed by Hoebe and colleagues Hoebe, K. et al. *Nat Immunol* 4, 1223-9 (2003). is not involved in the immune recognition of siRNA.

Non-target-specific downregulation of protein expression by siRNA-induced
type I IFN production is a major issue in RNA interference technology. Ways to
separate type I IFN inducing properties from the silencing activity of siRNA will
advance the application of siRNA. In our study, the siRNA TLR9.2 turned out to be

5   an excellent model to demonstrate that silencing activity and immunostimulatory
activity are two unrelated functional properties of siRNA. Dissection of both
functional activities was possible since the RNA sequence responsible for IFN-α
induction was found to be located on the sense strand and not the anti-sense strand.
By selected backbone modification (with locked nucleic acid; LNA) of certain regions

10   on the sense or the antisense strand of siRNA TLR9.2, we were able to generate
derivatives of siRNA TLR9.2 in which the immunostimulatory activity was abolished
while the silencing activity was maintained, and vice versa. This demonstrated that
the functional properties of siRNA can be changed to favor immunostimulation and/or
target mRNA silencing. Obviously, in addition to their effects on mRNA silencing,

15   certain siRNA can also have added therapeutic potential due to their potential
immunostimulatory properties, especially as it relates to use as an anti-infective or
anti-tumor therapeutic. The stimulatory sequence used in this study resulted in IFN-α
induction that was approximately 30 % of the maximal stimulation seen in PDC
(stimulus: CpG-A ODN 2216). Although it is unlikely that this stimulatory sequence

20   is optimal, our results with this RNA sequence *in vivo* highlight the potential for
further development of immunostimulatory siRNA molecules.

**Example 2: Synthesis of modified oligonucleotides for modulating
immunostimulation**

(a) RNA, 2'-O-Methyl-modified, 2'-deoxy-2'-fluoro-modified, and thioate-
25   modified oligonucleotides

The RNA molecules were synthesized on a 394 ABI machine using the
standard cycle written by the manufacturer with modifications to a few wait steps as
described below. The solid support was controlled pore glass (CPG; 500Å, Prime
Synthesis, Inc., Aston, PA, USA). The monomers were RNA phosphoramidites or 2'-
30   O-Methyl (2'-OMe) RNA phosphoramidites with standard protecting groups obtained
from Pierce Nucleic Acid Technologies (Pierce Milwaukee LLC, Milwaukee, WI,

USA) used at concentrations of 0.15 M in CH_3CN unless otherwise stated. Specifically the RNA phosphoramidites were 5'-O-dimethoxytrityl-N^6-benzoyl-2'-O-*t*butyldimethylsilyl-adenosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-dimethoxytrityl-N^2-isobutyryl-2'-O-*t*butyldimethylsilyl-guanosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-dimethoxytrityl-N^4-acetyl-2'-O-*t*butyldimethylsilyl-cytidine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite, 5'-O-dimethoxytrityl-2'-O-*t*butyldimethylsilyl-uridine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-dimethoxytrityl-N^6-benzoyl-2'-O-methyl-adenosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-dimethoxytrityl-N^2-isobutyryl-2'-O-methyl-guanosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-dimethoxytrityl- N^4-acetyl-2'-O- methyl-cytidine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite, and 5'-O-dimethoxytrityl-2'-O-methyl-uridine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite. The 2'-deoxy-2'-fluoro (2'-F) phosphoramidites, 5'-O-dimethoxytrityl-N4-acetyl-2'-deoxy-2'-fluoro-cytidine-3'-O-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite and 5'-O-dimethoxytrityl-2'-deoxy-2'-fluoro-uridine-3'-O-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite were obtained from Promega Corp. (Madison, WI, USA). 5'-O-dimethoxytrityl-N6-benzoyl-2'-deoxy-2'-fluoro-adenosine-3'-O-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite and 5'-O-dimethoxytrityl-N2-isobutyryl-2'-deoxy-2'-fluoro-guanosine-3'-O-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite were synthesized according to literature protocols (citations?). The coupling times were 10 min for all monomers. Details of the other reagents are as follows: activator, 5-ethyl thiotetrazole (0.25 M, Glen Research, Sterling, VI, USA); Cap A, 5% acetic anhydride/THF/pyridine (Glen Research, Sterling, VI, USA); Cap B: 10% N-methylimidazole/THF (Glen Research, Sterling, VI, USA); PO oxidation involved 0.02 M I_2/THF/H_2O (Glen Research, Sterling, VI, USA). For the PS-oxidation a solution of the Beaucage reagent (Chruachem Ltd, Glasgow, UK) in acetonitrile (1%) was used. The trityl group was removed on the synthesizer with 3% TCA/dichloromethane (Glen Research, Sterling, VI, USA).

(b) Synthesis of Oligonucleotides with LNA linkages

      The RNA molecules were synthesized on a 394 ABI machine using the standard cycle written by the manufacturer with modifications to a few wait steps as described below. The solid support was rA CPG (2 μmole, 520Å, Prime Synthesis, Inc., Aston, PA, USA, batch #CPG60N11RASN), rU CPG (G311103 is this a Prime Synthesis, Inc., Aston, PA, USA batch number as well?), LocA (2 μmole, Proligo LLC, Boulder, CO, USA, 40.0 μmoles/g, batch #225401), or LocT (2 μmole, Proligo LLC, Boulder, CO, USA, 39.0 μmoles/g, batch #224597). Loc designates an LNA building block. The monomers were either RNA phosphoramidites (Pierce Nucleic Acid Technologies, Pierce Milwaukee LLC, Milwaukee, WI, USA) or LNA phosphoramidites LocA^{Bz} (Proligo LLC, Boulder, CO, USA, cat. no. 223917), $^{5'DMT}_{CE}$Loc^{5me}C^{Bz} (Proligo LLC, Boulder, CO, USA, cat. no. 223816), $^{5'DMT}_{CE}$Loc^{5me}G^{iBu} (Proligo LLC, Boulder, CO, USA, cat. no. 223817), $^{5'DMT}_{CE}$LocT (Proligo LLC, Boulder, CO, USA, cat. no. 22818). All had standard protecting groups and were used at concentrations of 0.15 M in CH$_3$CN unless otherwise stated. Specifically the RNA phosphoramidites were 5'-O-dimethoxytrityl-N^6-benzoyl-2'-O-*t*butyldimethylsilyl-adenosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite (batch # FG0082), 5'-O-dimethoxytrityl-N^2-isobutyryl-2'-O-*t*butyldimethylsilyl-guanosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite (batch #FE0031), 5'-O-dimethoxytrityl-N^4-acetyl-2'-O-*t*butyldimethylsilyl-cytidine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite (batch # FF0046), and 5'-O-dimethoxytrityl-2'-O-*t*butyldimethylsilyl-uridine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite (batch #FF0036). The LNA phosphoramidites were 5'-O-dimethoxytrityl-N^6-benzoyl-adenosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) LNA phosphoramidite, 5'-O-dimethoxytrityl-N^2-isobutyryl-guanosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) LNA phosphoramidite, 5'-O-dimethoxytrityl-N^4-acetyl-5-methyl-cytidine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) LNA phosphoramidite, and 5'-O-dimethoxytrityl-5-methyl-thymidine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) LNA phosphoramidite.

      The coupling times were 10 mins for all monomers. Details of the other reagents are as follows: activator: 5-ethyl thiotetrazole (0.25 M, Glen Research,

Sterling, VI, USA); Cap A, 5% acetic anhydride/THF/pyridine (Glen Research,

Sterling, VI, USA); & Cap B, 10% N-methylimidazole/THF (Glen Research, Sterling,

VI, USA); PO oxidation involved 0.02M I$_2$/THF/H$_2$O (Glen Research, Sterling, VI,

USA). The trityl group was removed on the synthesizer with 3%

5   TCA/dichloromethane (Glen Research, Sterling, VI, USA).

Deprotection

After completion of synthesis the controlled pore glass (CPG) was transferred

to a screw cap sterile microfuge tube. The oligonucleotide was cleaved and

simultaneously the base and phosphate groups deprotected with 1.0 mL of a mixture

10   of ethanolic ammonia (1:3) for 5 hours at 55°C. The tube was cooled briefly on ice

and then the solution was transferred to a 5 mL centrifuge tube. The solid support was

washed three times using 0.25 mL of 50% CH$_3$CN in water and the washes were

added to the original solution. The tubes were cooled at -80°C for 15 min and the

solution was dried in a lyophilizer.

15   The white residue obtained was resuspended in 200 μL of triethylamine

trihydrofluoride and heated at 65°C for 1.5h to remove the TBDMS groups at the 2'-

position. The oligonucleotide was then precipitated in dry methanol (400 μL). The

liquid was removed carefully to yield a pellet at the bottom of the tube. Residual

methanol was removed in the speed vacuum to give the crude RNA as a white fluffy

20   material.

Purification

Samples were dissolved in 1mL RNase free water and quantitated at 260 nm.

The crude oligonucleotides were purified by denaturing gel electrophoresis (20%

acrylamide, 6 M urea). The purified oligonucleotides were desalted using Sephadex

25   G25M (Amersham Biosciences Corp, Piscataway, NJ, USA).

Tables 3-6 show the sequences of the so modified oligonucleotides that were

synthesized.

60

Determination of activity

Modified oligonucleotides were incubated with PDC as described above in
Example 1, and IFN-α production was measured. Table 7 shows the results obtained
for oligonucleotides having a base sequence identical to TLR9.2s except for the
5   terminal thymidines, but containing 2'-fluoro or 2'-O-methyl-modifications in
positions 1 and 2, 1, 2, 18, and 19, 1 through 4 and 16 through 19, and 16 through 19,
or a fully phosphorothioate-substituted backbone, respectively, expressed in % of the
IFN-α production measured for TLR9.2s. It is evident that the 2'-fluoro modification
generally has less effect on the immunostimulating activity of these oligonucleotides,
10   while 2'-O-methyl modifications almost completely abolish this activity. For the 2'-
fluoro modification, modifying only those nucleotides at the 5'-end, farthest away
from the 5'-GUCCUUCAA-3' (SEQ ID NO:1) motif, had the least effect, while
modifying 4 nucleotides on both ends severely diminished the immunostimulatory
activity of the oligonucleotide. Phosphorothioates moderately diminished the IFN-α
15   inducing activity.

Hence, if it seems desirable to modify an immunostimulatory oligonucleotide
of the invention, e.g. in order to protect it against nucleolytic degradation, this may be
achieved by introducing 2'-fluoro modifications, and preferably by introducing these
modifications in such a manner that the nucleotides of SEQ ID NO:1 remain
20   unmodified. However, if it is desired to generate an oligonucleotide comprising SEQ
ID NO:1, or 4 or more, 5 or more, 6 or more, 7 or more or 8 or more contiguous
nucleotides from SEQ ID NO:1, e.g. 5'-GUCC-3' (SEQ ID NO:2), 5'-GUCCU-3'
(SEQ ID NO:3), 5'-GUCCUU-3' (SEQ ID NO:4), 5'-GUCCUUC-3' (SEQ ID NO:5),
or 5'-GUCCUUCA-3' (SEQ ID NO:6), but immunostimulating activity is to be
25   avoided, then this may be achieved by introducing 2'-O-methyl modifications, and
preferably by introducing these modifications in such a manner that the nucleotides of
SEQ ID NO:1 are modified.

Table 1: siRNA sequences

Gene target (starting base)	Our no.	Sequence (sense / antisense)	Base composition				SEQ ID NO:
			A	G	U	C	
hTLR2 (29)	TLR2.1s	5'-AAGGAGACCUAUAGUGACUdTdT	8	11	11	8	SEQ ID NO:8
	TLR2.1as	5'-AGUCACUAUAGGUCUCCUUdTdT					SEQ ID NO:9
hTLR3 (71)	TLR3.1s	5'-GGAAAGGCUAGCAGUCAUCdTdT	10	9	9	10	SEQ ID NO:10
	TLR3.1as	5'-GAUGACUGCUAGCCUUUCCdTdT					SEQ ID NO:11
(331)	TLR3.2s	5'-ACUAGCUUGGAUGUAGGAUdTdT	8	11	11	8	SEQ ID NO:12
	TLR3.2as	5'-AUCCUACAUCCAAGCUAGUdTdT					SEQ ID NO:13
hTLR4 (1039)	TLR4.1s	5'-UACUUAGACUACUACCUCGdTdT	8	11	11	8	SEQ ID NO:14
	TLR4.1as	5'-CGAGGUAGUAGUCUAAGUAdTdT					SEQ ID NO:15
(1597)	TLR4.2s	5'-AUGGCUGGCAAUUCUUUCCdTdT	9	10	10	9	SEQ ID NO:16
	TLR4.2as	5'-GGAAAGAAUUGCCAGCCAUdTdT					SEQ ID NO:17
hTLR9 (1155)	TLR9.1s	5'-UGGACGGCAACGUUAUUAdTdT	8	11	11	8	SEQ ID NO:18
	TLR9.1as	5'-UAAUAACAGUUGCCGUCCAdTdT					SEQ ID NO:19
(1647)	TLR9.2s	5'-AGCUUAACCUGUCCUUCAAdTdT	8	11	11	8	SEQ ID NO:20
	TLR9.2as	5'-UUGAAGGACAGGUUAAGCUdTdT					SEQ ID NO:21
(1653)	TLR9.3s	5'-ACCUGUCCUUCAAUUACCAdTdT	8	11	11	8	SEQ ID NO:22
	TLR9.3as	5'-UGGUAAUUGAAGGACAGGUdTdT					SEQ ID NO:23
(2251)	TLR9.4s	5'-CUCAUUCACGGAGCUACCAdTdT	10	9	9	10	SEQ ID NO:24
	TLR9.4as	5'-UGGUAGCUCCGUGAAUGAGdTdT					SEQ ID NO:25
eGFP (435)	GFPs	5'-CUACAACAGCCACAACGUCdTdT	10	9	9	10	SEQ ID NO:26
	GFPas	5'-GACGUUGUGGCUGUUGUAGdTdT					SEQ ID NO:27

Numbers in parentheses indicate the nucleotide position at which the 5'- end of the sense strand of the siRNA matches the target sequence for either hTLR2 (NM_003264), hTLR3 (NM_003265), hTLR4 (NM_138554), hTLR9 (NM_017442), enhanced GFP.

Table 2: Single-Stranded RNA Oligonucleotides

Original sequence	Name[1]	Sequence 5' → 3'[2]	SEQ ID NO:
TLR9.2s		AGCUUAACCUGUCCUUCAA	SEQ ID NO:28
	L8A	AAAAAAAACUGUCCUUCAA	SEQ ID NO:29
	L9A	AAAAAAAAAUGUCCUUCAA	SEQ ID NO:30
	L10A	AAAAAAAAAAGUCCUUCAA	SEQ ID NO:31
	L11A	AAAAAAAAAAAUCCUUCAA	SEQ ID NO:32
	L12A	AAAAAAAAAAAACCUUCAA	SEQ ID NO:33
	R3A	AGCUUAACCUGUCCUUAAA	SEQ ID NO:34
	R4A	AGCUUAACCUGUCCUAAAA	SEQ ID NO:35
	R5A	AGCUUAACCUGUCCAAAAA	SEQ ID NO:36
	R8A	AGCUUAACCUGAAAAAAAA	SEQ ID NO:37
	DR	UGUCCUUCAAUGUCCUUCAA	SEQ ID NO:38
	19U	AGCUUAACCUGUCCUUCAU	SEQ ID NO:39
	18/19UU	AGCUUAACCUGUCCUUCUU	SEQ ID NO:40
	hairpin	AGCUUAACCUGUCCUUCAA-CUACACAAAUUGAAGGAC AGGUUAAGCU	SEQ ID NO:41
	5'F	F-AGCUUAACCUGUCCUUCAA	SEQ ID NO:42
	3'F	AGCUUAACCUGUCCUUCAA-F	SEQ ID NO:43
	5'LNA	AGCUUAACCUGUCCUUCAA	SEQ ID NO:44
	3'LNA	AGCUUAACCUGUCCUUCAA	SEQ ID NO:45
	5'3'LNA	AGCUUAACCUGUCCUUCAA	SEQ ID NO:46
	16mer	UUAACCUGUCCUUCAA	SEQ ID NO:47
	12mer	AACCUGUCCUUCA	SEQ ID NO:48
TLR9.2as		UUGAAGGACAGGUUAAGCU	SEQ ID NO:49
	5'LNA	UUGAAGGACAGGUUAAGCU	SEQ ID NO:50
	3'LNA	UUGAAGGACAGGUUAAGCU	SEQ ID NO:51
	5'3'LNA	UUGAAGGACAGGUUAAGCU	SEQ ID NO:52
	polyA	AAAAAAAAAAAAAAAAAAA	SEQ ID NO:53
	polyU	UUUUUUUUUUUUUUUUUUU	SEQ ID NO:54

[1] L8A: read „8 A from left" (5' end); R3A: read „3 A from right" (3' end); DR: read „double right" (sequence containing the 3' 9mer sequence of 9.2 sense two times); 19U: read „base at position 19 replaced by U"; 18/19UU read „bases at position 18 and 19 replaced by U";

[2] underlined: 3' 9mer sequence of TLR9.2s; F: FITC; bold: LNA modification.

Table 3: Oligonucleotides containing ribofluoro modifications

Our No.	Sequence	D/tube	Mass	OD/mg	Extn coeff. e260*10^{-3}	SEQ ID NO:
2792	fAfGfCfUUAACCUGUCCUUCAA	2	5950.57	29.8	177	SEQ ID NO:55
2793	fAfGCUUAACCUGUCCUUCfAfAdT	3	6254.77	29.6	185	SEQ ID NO:56
2794	fAfGfCfUUAACCUGUCCUUfCfAfAdT	2	6262.77	29.6	185	SEQ ID NO:57
2795	AGCUUAACCUGUCCUUfCfAfAdT	3	6254.77	29.6	185	SEQ ID NO:58
2796	fUfUfGfAAGGACAGGUUAAGCU	3	6133.73	32.5	199	SEQ ID NO:59
2797	fUfUfGfAAGGACAGGUUAAGfCfUdT	2	6437.93	32.2	207	SEQ ID NO:60
2798	fUfUfGfAAGGACAGGUUAfAfGfCfUdT	2	6445.93	32.1	207	SEQ ID NO:61
2799	UUGAAGGACAGGUUAfAfGfCfUdT	2	6437.93	32.2	207	SEQ ID NO:62

Table 4: Oligonucleotides containing phosphorothioates

Our No.	Sequence	OD/tube	Expected Mass	OD/mg	Extn coeff. e260*10-3	SEQ ID NO:
2808	AsGsCsUsAsAsCsCsUsGsUsCsCsUsUsCsAsA	2	6230.57	28.4	177	SEQ ID NO:63
2809	UsUsGsAsAsGsGsAsCsAsGsGsUsUsAsAsGsCsU	2	6413.73	31.03	199	SEQ ID NO:64

Table 5: Oligonucleotides containing 2'-O-methyl modifications

Our No.	Sequence	OD/tube	Expected Mass	OD/mg	Extn coeff. e260*10^{-3}	SEQ ID NO:
2800	oAoGoCoUUAACCUGUCCUUCAA	3	5998.69	29.6	177	SEQ ID NO:65
2801	oAoGCUUAACCUGUCCUUCoAoA	3	5998.69	29.6	177	SEQ ID NO:66
2802	oAoGoCoUUAACCUGUCCUoUoCoAoA	3	6054.81	29.28	177	SEQ ID NO:67
2803	AGCUUAACCUGUCCUoUoCoAoA	3	5998.69	29.55	177	SEQ ID NO:68
2804	oUoUoGoAAGGACAGGUUAAGCU	3	6181.85	32.20	199	SEQ ID NO:69
2805	UoUGAAGGACAGGUUAAGoCoU	3	6181.85	32.20	199	SEQ ID NO:70
2806	oUoUoGoAAGGACAGGUUAoAoGoCoU	3	6237.97	31.91	199	SEQ ID NO:71
2807	UUGAAGGACAGGUUAoAoGoCoU	3	6181.85	32.20	199	SEQ ID NO:72
2869	AGoCoUoUAAoCoCoUGoUoCoCoUoUoUooCAA	2	6110.93	29.2	177	SEQ ID NO:73
2870	oAGoCUoUoACCoUoCoUoGUoCCoUUoCAoA	2	6082.87	29.1	177	SEQ ID NO:74
2871	AoGCoUUoAAoCCoUoGoUCoCUUoUoCoAA	2	6068.84	29.2	177	SEQ ID NO:75
2872	AGCUoUoAACCoUGUCCUUoCoAA	2	5984.66	29.6	177	SEQ ID NO:76
2873	oUoUGAAGGAoCAGGoUoUAAGoCoU	2	6223.94	32.0	199	SEQ ID NO:77
2874	oUUoGAoAoGGoAoCAoGGoUUoAAoGCU	2	6251.64	31.8	199	SEQ ID NO:78
2875	UoUGoAAoGGoACoAGoGUoUAoAGoCU	2	6266.03	31.8	199	SEQ ID NO:79
2876	UoUGAAGGAoCAGGUUoUAAGCU	2	6167.82	32.3	199	SEQ ID NO:80

Table 6: Oligonucleotides containing Locked Nucleotides

Our No.	Sequence	Expected Mass	Experimen-tal mass	OD/mg	SEQ ID NO:
2784	ALGLm5CLTLUAACCUGUCCUUCAA	6018.82	6018.05	91.6	SEQ ID NO:81
2785	AGCUUAACCUGUCCUUTLm5CLALAL	6018.32	6017.99	84.84	SEQ ID NO:82
2786	ALGLm5CLTLUAACCUGUCCUUTLm5CLALAL	6095.09	6093.98	94.79	SEQ ID NO:83
2787	ALGLCUUAACCUGUCCUUCALAL	5990.63	5989.88	92.66	SEQ ID NO:84
2788	TLTLGLALAGGACAGGUUAAGCU	6201.96	6201.04	90.6	SEQ ID NO:85
2789	UUGAAGGACAGGUUAALGLm5CLTL	6201.96	6200.97	92.22	SEQ ID NO:86
2790	TLTLGLALAGGACAGGUUAALGLm5CLTL	6278.23	6277.1	93.18	SEQ ID NO:87
2791	TLTLGAAGGACAGGUUAAGm5CLTL	6230.15	6229.29	92.2	SEQ ID NO:88

In Tables 3-6, strands are shown written 5' to 3'. dT means 2'-deoxy thymidine, a leading lower case "s" indicates a 5'-phosphorothioate group. Locked nucleic acids are indicated by a trailing "L". A leading lower case "d" indicates a deoxy residue. A leading "o" indicates a 2'-O-methyl modified nucleotide. A leading "f" indicates a 2'-fluoro nucleotide. "T" indicates a 5-methyl-uridine and "m5C" indicates a 5-methyl-cytidine. Six of the sequences in the fluoro set end in "dT" overhangs as the appropriate CPG containing ribofluoro nucleosides were not available at that time. These are 2793, 2794, 2795, 2797, 2798, and 2799. Extn coeff.e260*10⁻³ is the molar extinction coefficient of the oligonucleotide at 260 nm wavelength divided by (1000 x l/(mol x cm).

Table 7: Interferon production in PDC cells incubated with modified oligonucleotides derived from RNA9.2s

Our No.	IFN-α production (in % of control)
TLR9.2s	100% (4.5 ng/ml IFN-α)
2792	72%
2793	56%
2794	11%
2795	26%
2800	9%
2801	11%
2802	12%
2803	8%
2808	64%

Table 8: Gene transcripts comprising a sequence identical or complementary to SEQ ID NO:1

spectrin, alpha, erythrocytic 1 (elliptocytosis 2)
spectrin, beta, non-erythrocytic 1 (SPTBN1), transcript variant 1 and 2
ryanodine receptor 1 (skeletal) (RYR1)
WD repeat domain 9 (WDR9), transcript variant 2
WD repeat domain 9 (WDR9), transcript variant 1
phosphoglycerate kinase 1 (PGK1)
SEC11-like 3 (S. cerevisiae) (SEC11L3)
SET domain, bifurcated 1 (SETDB1)
HBS1-like (S. cerevisiae) (HBS1L)
activation-induced cytidine deaminase (AICDA)
cholinergic receptor, muscarinic 3 (CHRM3)
transmembrane protein 1 (TMEM1), transcript variant 1 and 2
SFRS protein kinase 2 (SRPK2), transcript variant 1
step II splicing factor SLU7 (SLU7)
myosin, heavy polypeptide 7, cardiac muscle, beta
keratin, hair, acidic, 7 (KRTHA7)
keratin, hair, acidic, 8 (KRTHA8)
inter-alpha (globulin) inhibitor H3 (ITIH3)

integrin, alpha 4 (antigen CD49D, alpha 4 subunit of
integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2
cleavage stimulation factor, 3' pre-RNA, subunit 2
cubilin (intrinsic factor-cobalamin receptor) (CUBN)
cytochrome P450, family 2, subfamily F, polypeptide 1
UDP-N-acetyl-alpha-D-galactosamine:polypeptide-N-acetylgalactosaminyltransferase 8 (GalNAc-T8) (GALNT8)
UDP-N-acetyl-alpha-D-galactosamine:polypeptide-N-acetylgalactosaminyltransferase 7 (GalNAc-T7) (GALNT7)
E74-like factor 2 (ets domain transcription factor) (ELF2), transcript variant 1 and 2
endogenous retroviral family W, env(C7), member 1 (syncytin) (ERVWE1)
solute carrier family 7 (cationic amino acid transporter, y+ system), member 6 (SLC7A6)
AT-binding transcription factor 1 (ATBF1)
gasdermin-like (GSDML)
sodium channel, nonvoltage-gated 1, gamma (SCNN1G)
zinc finger and SCAN domain containing 1 (ZSCAN1)
ubiquitin specific protease 54 (USP54)
vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR)
CD84 antigen (leukocyte antigen) (CD84)
alpha-kinase 3 (ALPK3)
interferon, alpha 2 (IFNA2)
zinc finger protein 452 (ZNF452)
SNF7 domain containing 2 (SNF7DC2)
platelet/endothelial cell adhesion molecule (CD31 antigen) (PECAM1)
hypothetical protein DKFZp547K1113 (DKFZp547K1113)
repressor of estrogen receptor activity (REA)
fibrous sheath interacting protein 1 (FSIP1)
neural cell adhesion molecule 1 (NCAM1)
immunoglobulin superfamily, member 4 (IGSF4)
protein inhibitor of activated STAT, 2 (PIAS2), transcript variant alpha and beta
F-box protein 21 (FBXO21), transcript variant 1 and 2
F-box protein 22 (FBXO22), transcript variant 2
Leucine-zipper-like transcription regulator 1 (LZTR1)
Nuclear receptor subfamily 1, group H, member 2 (NR1H2)
UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1 (B4GALT1)
CSRP2 binding protein (CSRP2BP), transcript variant 1 and 2

cleavage stimulation factor, 3' pre-RNA, subunit 1, 50kDa (CSTF1)
solute carrier organic anion transporter family, member 4A1 (SLCO4A1)
serine/threonine kinase 6 (STK6), transcript variant 3, 4, 5 and 6
zinc finger protein 295 (ZNF295)
FLJ16231 protein (FLJ16231)
zinc finger protein 646 (ZNF646)
mediator of RNA polymerase II transcription, subunit 12 homolog (yeast)-like (MED12L)
olfactory receptor, family 2, subfamily W, member 1 (OR2W1)
interferon, alpha 7 (IFNA7)
apolipoprotein B (including Ag(x) antigen) (APOB)
syndecan binding protein (syntenin) (SDCBP), transcript variant 1, 2, 3, 4 and 5
erbb2 interacting protein (ERBB2IP), transcript variant 2 and 7
fibroblast growth factor 23 (FGF23)
MARCKS-like 1 (MARCKSL1)
v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian) (SRC), transcript variant 1 and 2
v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian) (SRC), transcript variant 2
CUB and Sushi multiple domains 2 (CSMD2)
family with sequence similarity 49, member B (FAM49B)
pyruvate dehydrogenase kinase, isoenzyme 1 (PDK1), nuclear gene encoding mitochondrial protein
neurotrophic tyrosine kinase, receptor, type 3 (NTRK3), transcript variant 3
ret finger protein 2 (RFP2), transcript variant 2, 3 and 4
KIAA1950 protein (KIAA1950)
collagen, type XIV, alpha 1 (undulin) (COL14A1)
tektin 1 (TEKT1)
zinc finger protein 31 (KOX 29) (ZNF31)
Leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4)
protein kinase, DNA-activated, catalytic polypeptide (PRKDC)
scavenger receptor class B, member 1 (SCARB1)

WHAT IS CLAIMED IS:

1. A method of stimulating an immune response in a mammal comprising the step of administering to said mammal an oligonucleotide agent consisting of, consisting essentially of, or comprising a sequence of 4 or more, 5 or more, 6 or more, 7 or more or 8 or more contiguous nucleotides from SEQ ID NO:1, preferably taken from the 5' end of this sequence.

2. The method of claim 1, wherein the oligonucleotide agent consists of, consists essentially of, or comprises SEQ ID NO:1, or a sequence that differs by not more than 1 or not more than 2 nucleotides from SEQ ID NO:1.

3. The method of claim 1, wherein the oligonucleotide agent consists of, consists essentially of, or comprises SEQ ID NO:1.

4. The method of claim 1, wherein said sequence is chosen from the group of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

5. The method of any of the preceding claims, wherein the oligonucleotide is an iRNA agent.

6. The method of any of the preceding claims, wherein said agent is a single stranded RNA agent.

7. A method of making an oligonucleotide agent so as to avoid stimulating an immune response in a mammal comprising the step of eliminating from a potential agent pool any agent that comprises a sequence of 4 or more, 5 or more, 6 or more, 7 or more or 8 or more contiguous nucleotides from SEQ ID NO:1, preferably taken from the 5' end of this sequence, or a sequence that differs from SEQ ID NO:1 by not more than one or not more than 2 nucleotides.

8. The method of claim 7, wherein the oligonucleotide is an iRNA agent.

9. The method of claim 7, wherein said agent is a single stranded RNA agent.

70

10. An isolated oligonucleotide agent for inducing an immune response consisting of, consisting essentially of, or comprising a sequence of 4 or more, 5 or more, 6 or more, 7 or more or 8 or more contiguous nucleotides from SEQ ID NO:1, preferably taken from the 5' end of this sequence.

5   11. The isolated oligonucleotide agent of claim 10, wherein the oligonucleotide agent consists of, consists essentially of, or comprises SEQ ID NO:1, or a sequence that differs by not more than 1 or not more than 2 nucleotides from SEQ ID NO:1.

12. The isolated oligonucleotide agent of claim 10, wherein the oligonucleotide agent consists of, consists essentially of, or comprises SEQ ID NO:1.

10   13. The isolated oligonucleotide of claim 10, wherein said sequence is chosen from the group of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

14. The oligonucleotide agent of any one of claims 10 to 12, wherein the oligonucleotide agent is an iRNA agent.

15   15. The oligonucleotide agent of any one of claims 10 to 12, wherein said agent is a single stranded RNA agent.

16. The isolated oligonucleotide of any one of claims 10 to 15, further comprising at least one 2'-fluoro-modified nucleotide, wherein the 2'-fluoro-modified nucleotide is not part of a sequence of 4 or more contiguous nucleotides from SEQ ID NO:1.

20   17. A method of making an oligonucleotide agent so as to induce an immune response in a mammal, comprising the step of adding to a potential agent pool any agent that comprises 4 or more, 5 or more, 6 or more, 7 or more, or 8 or more contiguous nucleotides of SEQ ID NO:1, or a sequence that differs from SEQ ID NO:1 by not more than one or not more than 2 nucleotides.

25   18. The method of claim 17, wherein the oligonucleotide agent is an iRNA agent.

19. The oligonucleotide agent of claim 17, wherein said agent is a single stranded RNA agent.

20. A method of concomitantly inhibiting the expression of a gene and inducing an immune response in a mammal, comprising administering to said mammal an iRNA agent comprising a sequence of 4 or more, 5 or more, 6 or more, 7 or more, or 8 or more contiguous nucleotides of SEQ ID NO:1, preferably taken from the 5' end of this sequence, or a sequence that differs from SEQ ID NO:1 by not more than one or not more than 2 nucleotides.

21. The method of claim 20, wherein said sequence is chosen from the group of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6.

22. A method of evaluating an iRNA agent comprising:

a. providing a candidate iRNA agent;

b. reviewing the candidate iRNA agent to determine if it includes SEQ ID NO:1 or a sequence that differs from SEQ ID NO:1 by 1 or 2 nucleotides.

23. The method of claim 22, further comprising modifying the iRNA agent to remove the sequence of SEQ ID NO:1 or the sequence that differs from SEQ ID NO:1 by 1 or 2 nucleotides.

24. A pharmaceutical composition comprising an oligonucleotide of any one of claims 10 to 12 and a pharmaceutically acceptable carrier.

25. The pharmaceutical composition of claim 24, wherein he pharmaceutical composition is a vaccine.

26. A method of making an oligonucleotide agent so as to avoid stimulating an immune response in a mammal, wherein said oligonucleotides comprises a sequence of 4 or more, 5 or more, 6 or more, 7 or more or 8 or more contiguous nucleotides from SEQ ID NO:1, comprising providing the oligonucleotide agent

72

in such manner that it contains at least 2, or at least 4, 2'-*O*-methyl modified nucleotides.

27. The method of claim 26, wherein at least one, or at least two, of the 2'-*O*-methyl-modified nucleotides is part of a sequence of 4 or more contiguous nucleotides from SEQ ID NO:1
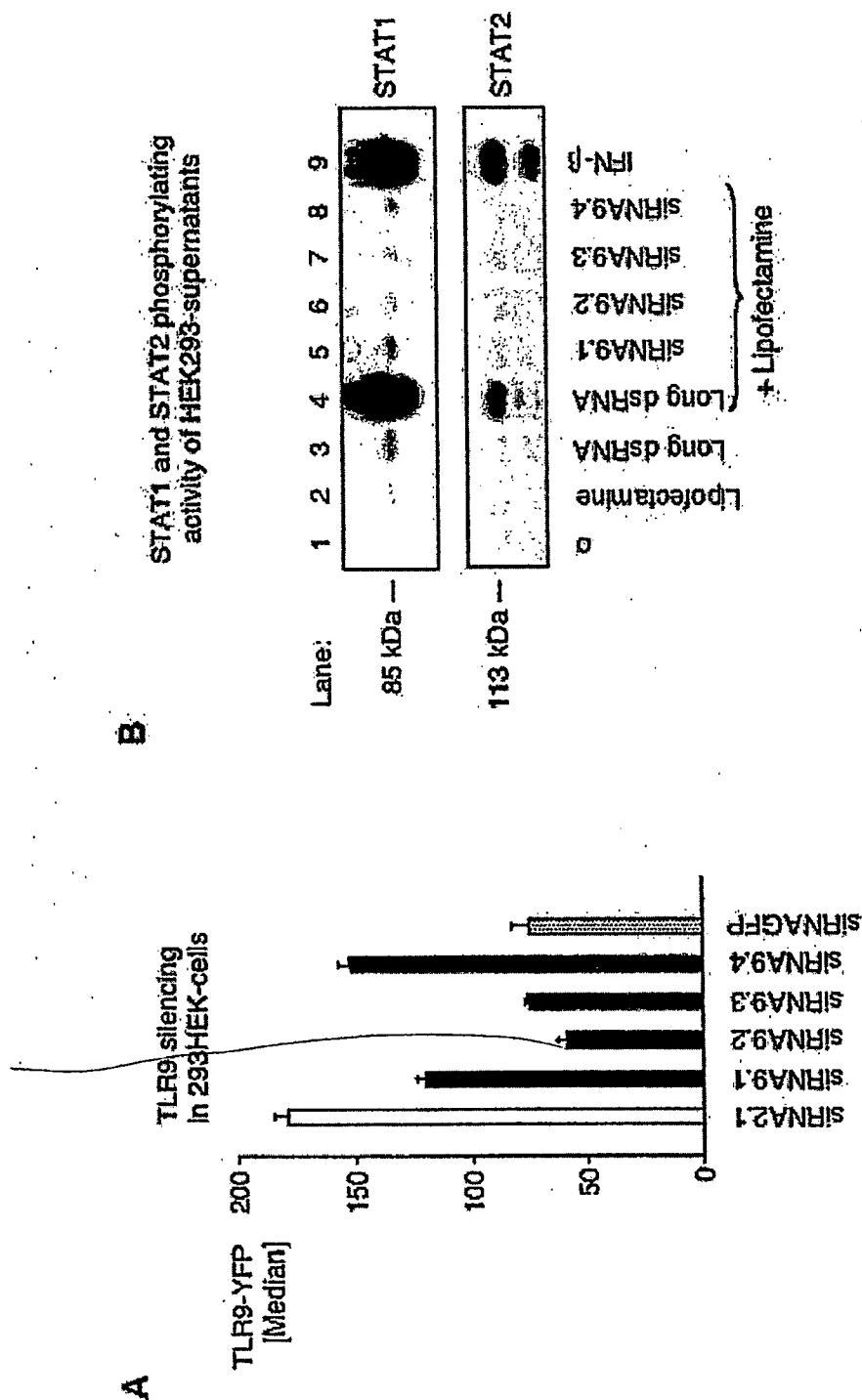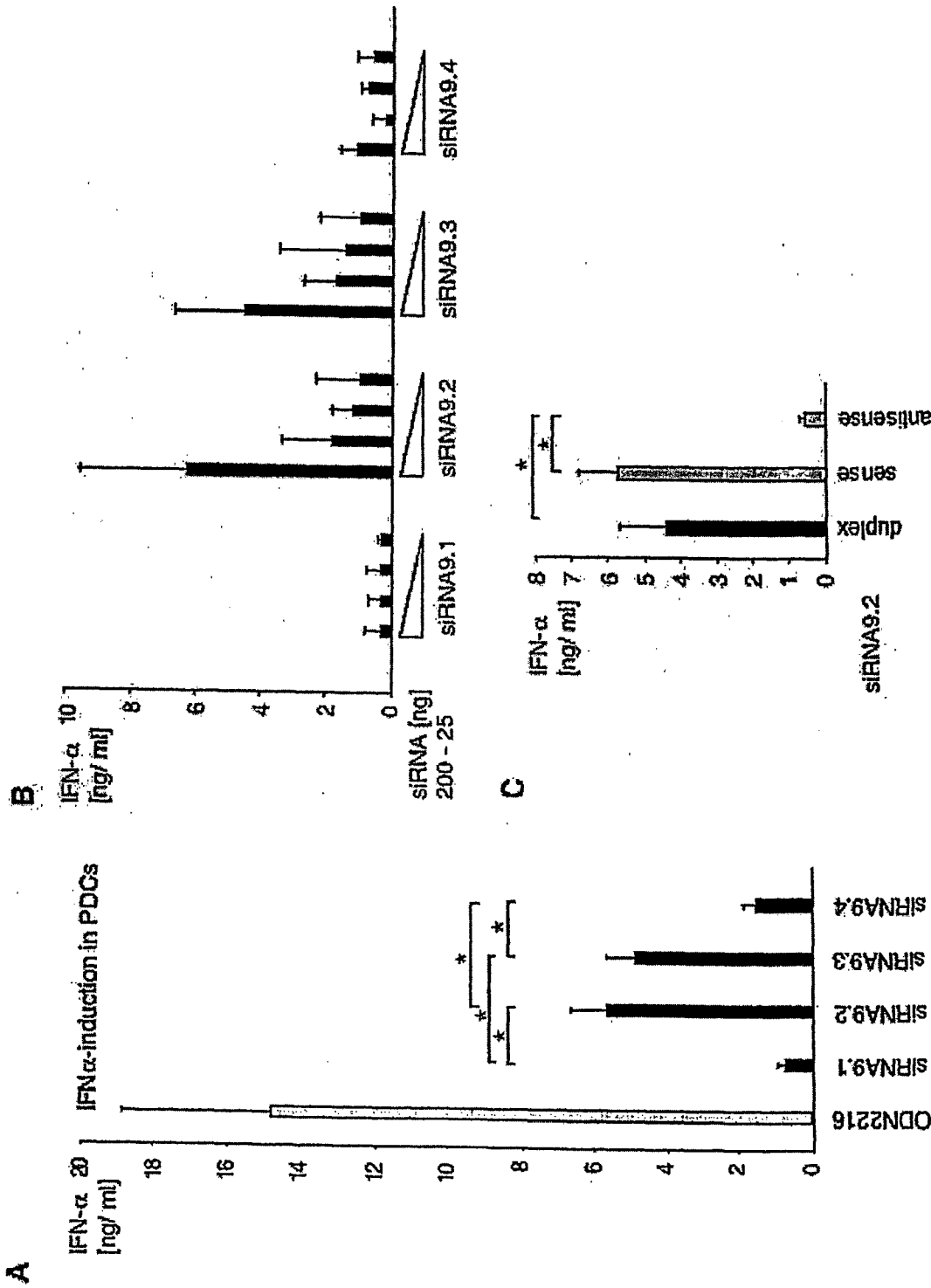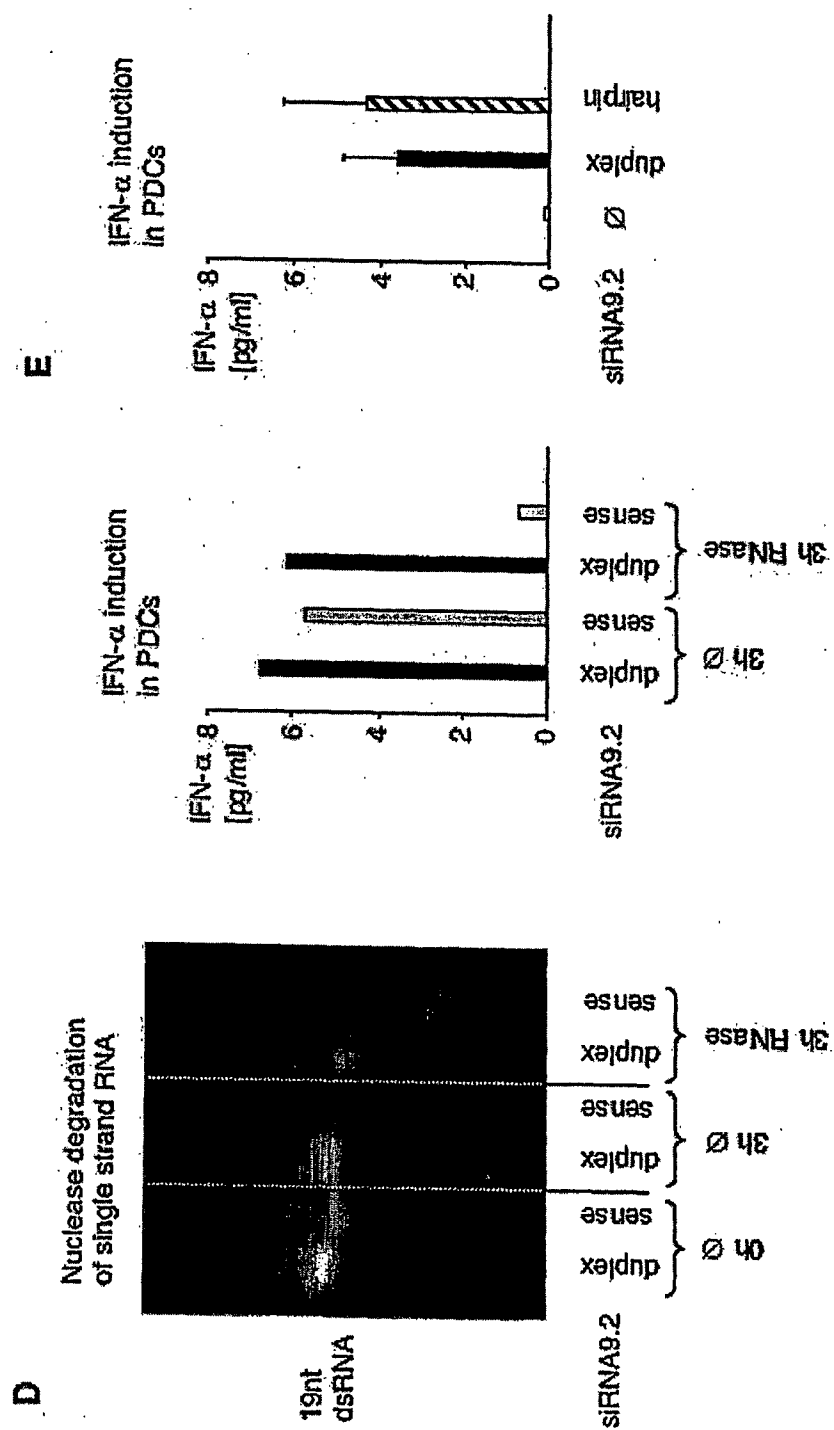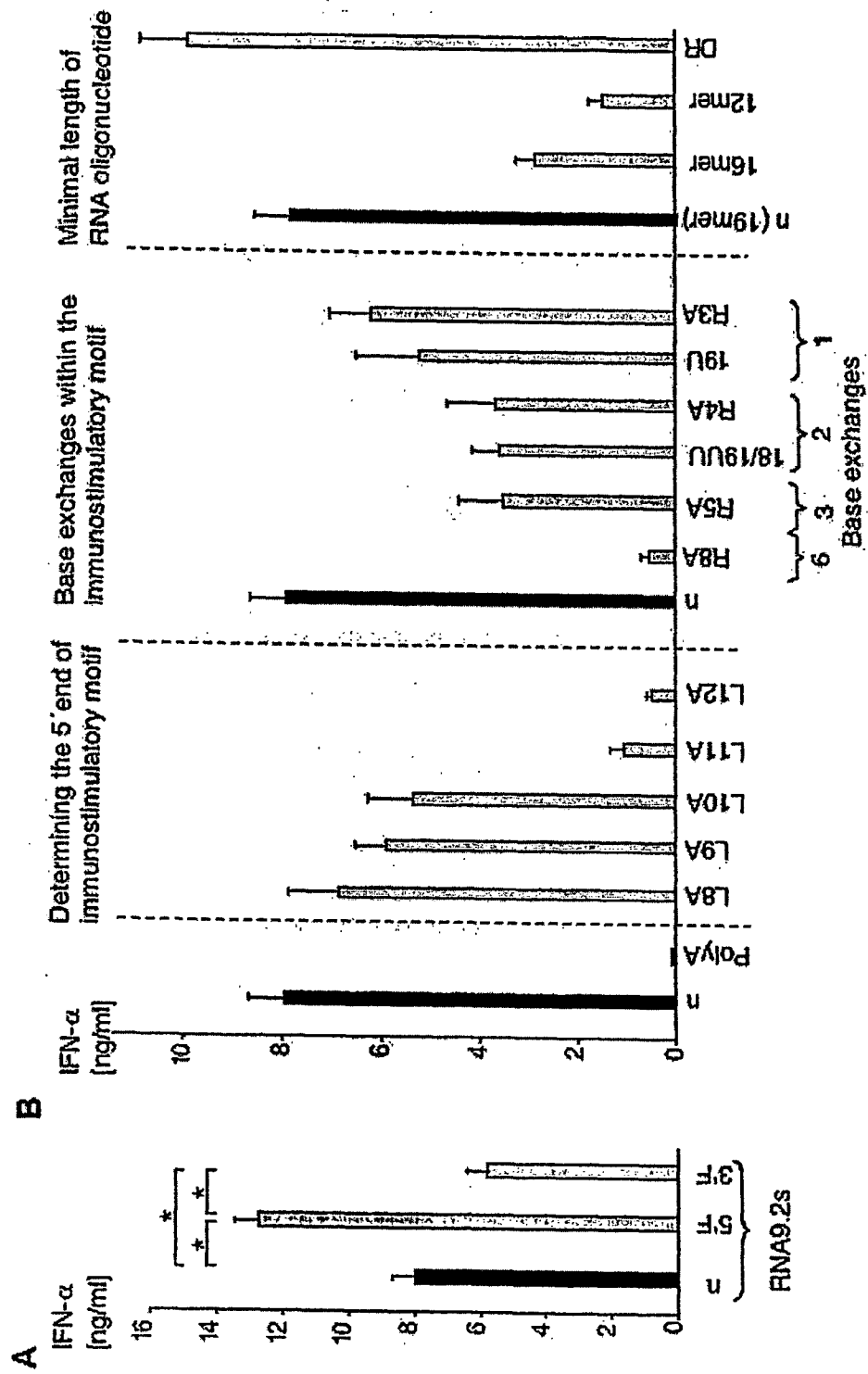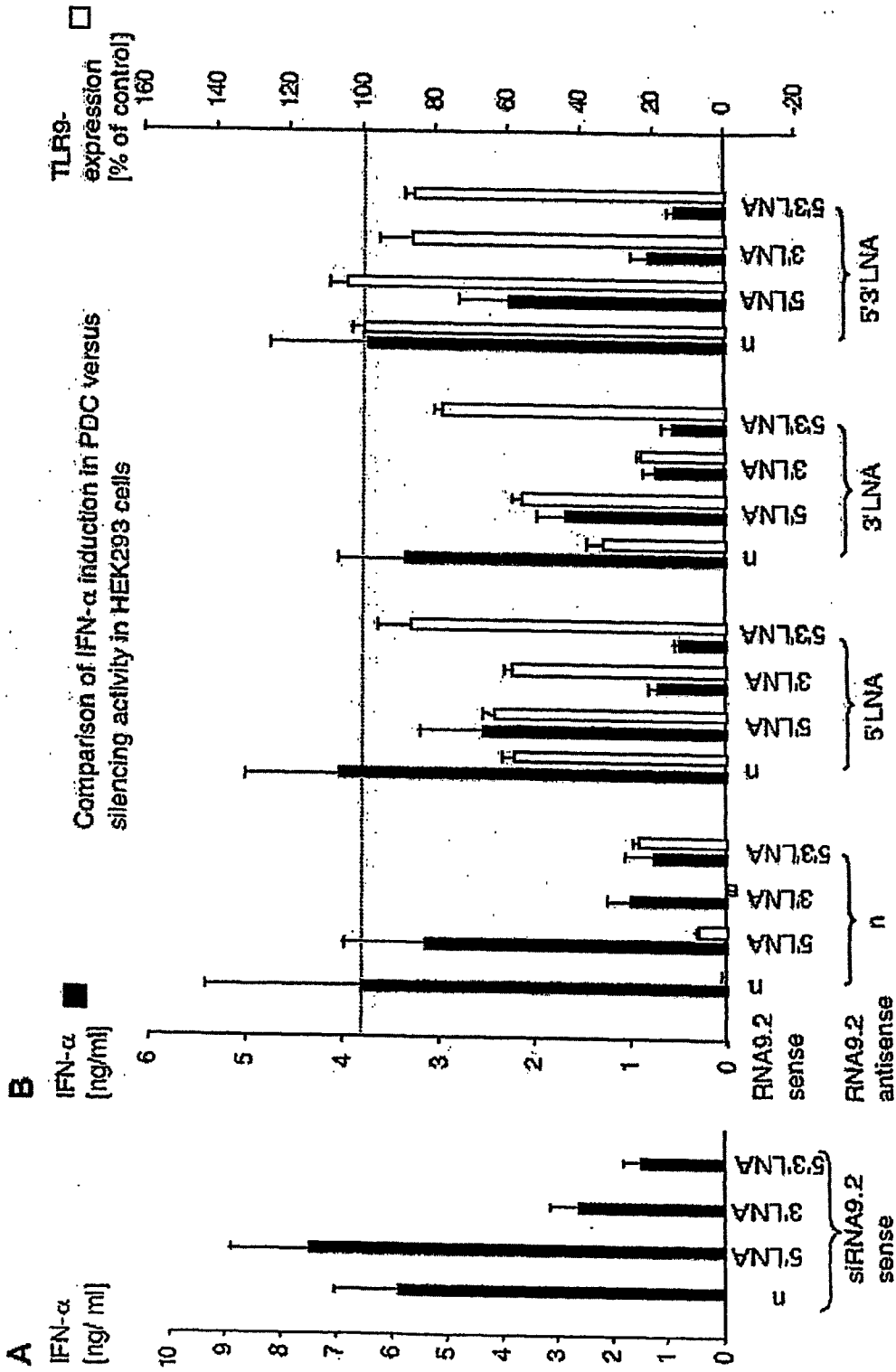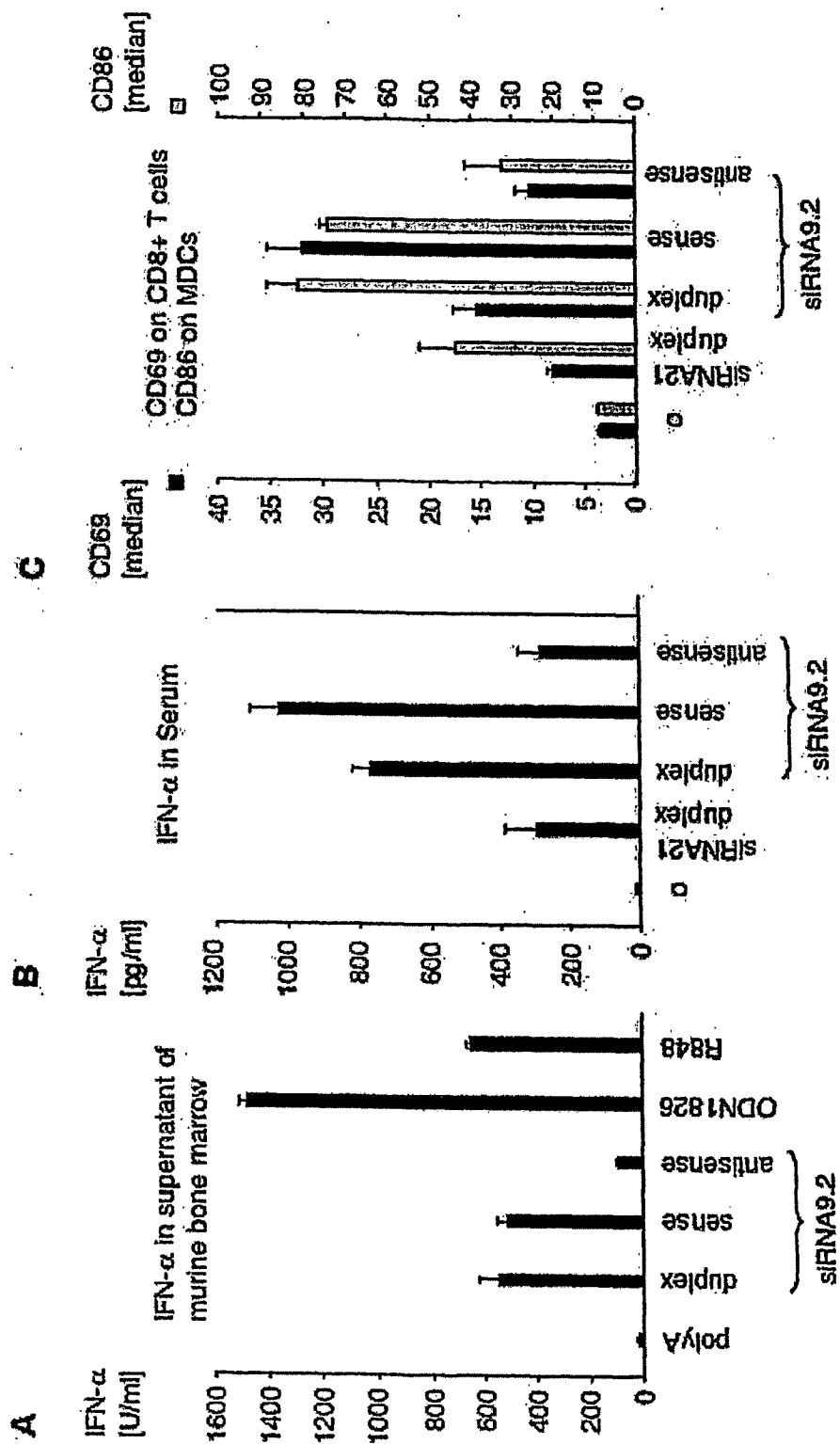
5

Fig. 1

FIG. 2

Fig. 2

Fig. 3

Fig. 4

FIG. 5

Fig. 5

Fig. 6

Fig. 6